OCT 22 1920

VOLUME V NUMBER 5

JOURNAL OF BACTERIOLOGY

OFFICIAL ORGAN OF THE SOCIETY OF AMERICAN BACTERIOLOGISTS

SEPTEMBER, 1920

EDITOR-IN-CHIEF

C.-E. A. WINSLOW

It is characteristic of Science and Progress that they continually open new fields to our vision.—PASTEUR.

PUBLISHED BI-MONTHLY

WILLIAMS & WILKINS COMPANY

BALTIMORE, U. S. A.

THE CAMBRIDGE UNIVERSITY PRESS

FETTER LANE, LONDON: E. C.

Entered as second-class matter April 9, 1918, at the postoffice at Baltimore, Maryland, under the act of March 3, 1879. Acceptance for mailing at special rate of postage provided for in Section 1103, Act of October 3, 1917. Authorized on July 13, 1918.

Copyrighted, 1920, Williams and Wilkins Company

Price, net postpaid { $5.00 per volume, United States, Mexico, Cuba
$5.25 per volume, Canada
$5.50 per volume, other countries

GASTRON
AT THE THRESHOLD

To gastric juice Nature assigns the rôle of a powerful antiseptic.

GASTRON, an extract of the actual tissue juice of the entire stomach mucosa, contains all the soluble, the activable constituents of the gastric juice itself in a carminative agreeable solution with 0.25 per cent hydrochloric acid, organically bound.

At the threshold of the digestive tract Gastron may be utilized as a physiological recourse against fermentative dyspepsia, to supplement and fortify impaired digestion.

Gastron is alcohol free.

Fairchild Bros. & Foster
NEW YORK

LaMotte Standards
GUARANTEED

Section 1.—Standardized Indicator dyes. Covering a wide range of H-ion concentration. Supplied in dry form and in sterile stock solutions. Each Indicator is standardized in strict accordance with the specifications of Clark & Lubs. (Jr. Bact., Vol. II, Nos. 1, 2, 3, 1917.)

Common Name	Color Change	Ps Value
Thymol Blue (acid range)	red-yellow	1.2-2.8
Methyl Orange	red-yellow	3.1-4.4
Bromphenol Blue	yellow-blue	3.0-4.6
Resorcin Blue	yellow-blue	4.6-6.0
Methyl Red	pink-blue	4.8-6.2
Bromcresol Purple	red-yellow	5.4-6.8
Litmus (special)	yellow-purple	5.5-7.5
Brom-thymol Blue	red-blue	6.2-7.6
Phenol Red	yellow-blue	6.8-8.2
Cresol Red	yellow-red	7.0-7.6
Thymol Blue (alkaline range)	yellow-red	8.0-9.6
Cresol-phthalein	yellow-blue	8.2-9.8
Phenol-phthalein	colorless-red	8.3-10.0
	colorless-red	9.4-11.0

Section 2-B.—Specially prepared and standardized Buffer salts and solutions. Buffer mixtures may be obtained in series covering any particular range of H-ion concentration from Ps 1.0 to 10.0.

Standardized Buffer Solutions (M/5)

Potassium Phosphate	Potassium Phthalate	Sodium Hydroxide (CO_2 free)
Potassium Chloride	Hydrochloric Acid	Di-Sodium Phosphate, H_2O

A large number of general synthetic and purified compounds are manufactured by us and information concerning them may be obtained by addressing

LA MOTTE CHEMICAL PRODUCTS CO.
"Standards Department"
13 W. Saratoga St. Baltimore, Md.

ERRATUM

J. Bact. **5**, 231. The Production of Hydrogen Sulphide by Bacteria. John T. Myers. From the Department of Hygiene and Bacteriology, the University of Chicago.

A portion of the work recorded in this paper was done in the laboratories of the University of Nebraska, College of Medicine, Department of Pathology and Bacteriology, Omaha, Nebraska.

THE FATE OF BACTERIA OF THE COLON-TYPHOID GROUP ON CARBOHYDRATE MEDIA

O. ISHII

From the Research Laboratory, Western Pennsylvania Hospital, Pittsburgh, Pennsylvania

Received for publication April 7, 1920

In any study of the fermentation reactions of the colon-typhoid group on carbohydrate culture media, it is customary for bacteriologists to make observations as to the quantity of acid, alkali, or gas formation for an unlimited number of days, sometimes for two weeks, or one month, or even for a much longer period. In this study we have found, however, that the organisms of the colon-typhoid group do not live as long as this on certain sugar culture media.

The technique used was as follows. The culture media containing the sugar were made with 1 per cent peptone plus 0.5 per cent sodium chloride, and they were sterilized once in the autoclave under 15 pounds pressure for fifteen minutes; this does not interfere with the differential fermentation reaction of these sugars, as we always have been able to differentiate quite satisfactorily the several strains of the colon-typhoid group with the above method.

Stock cultures were used and fresh subcultures were made in broth culture media. From the latter the sugar culture media were inoculated. The culture tubes were closed with cotton stoppers and kept in an incubator at 37°C. They were examined every day by the streak method on the plain agar plate, also in broth media, one loopful of the bacterial culture being taken from the sugar medium to determine whether or not the organisms were still living.

The results show that the period of life of the colon-typhoid group on certain of the different sugar cultures is short and on

others long (just the same results were obtained with or without an indicator, either litmus or china blue).

In the glucose-peptone water cultures in most cases, *Bact. typhi* died in three to five days, *Bact. paratyphi A* in three to five days, *Bact. paratyphi B* in three to six days, *Bact. enteritidis* in five to seven days, *Bact. suipestifer* in seven days, *Bact. coli* in six to seven days, *Proteus* in seven days and *Bact. dysenteriae* in three to seven days; but all strains of *Bact. pyocyaneus* still survived after thirty days' growth. The above results with *Ps. pyocyanea* were obtained with strains received from Dr. C.-E A. Winslow, and from Dr. Oscar Teague, Medical School of Columbia University of New York; a few strains, which we have isolated from feces, however, died within ten days in glucose broth, while others survived more than thirty days. It would seem that there are a number of different types of *Ps. pyocyanea* with different biological properties.

In mannitol peptone water cultures, in most cases, *Bact. typhi* died in four to six days, *Bact. paratyphi A* in four to five days, *Bact. paratyphi B* in five to eight days, *Bact. enteritidis* in about eight days, *Bact. suipestifer* in about eight days, *Bact. coli* in five to eight days, *Bact. dysenteriae* in five to ten days (with the exception of one which survived for over thirty days); *Proteus* and *Bact. pyocyaneus* were still alive after thirty days. One special strain of *Bact. dysenteriae* Shiga # T (obtained direct from Dr. Shiga, Japan), was still alive after thirty days, though another strain of Shiga no. 1, was dead in ten days. This is very peculiar, since culturally both were otherwise the same and there was no acid fermentation in either case.

In the lactose peptone water cultures of all the strains of *Bact. typhi, Bact. paratyphi A, Bact. paratyphi B, Bact. enteritidis, Bact. suipestifer, Proteus, Bact. pyocyaneus* and of all of the *Bact. dysenteriae* group, none had died within one month, but all strains of the *Bact. coli* group died within five to eighteen days.

In the sucrose peptone water cultures of all the strains used of *Bact. typhi, Bact. paratyphi A and B, Bact. enteritidis, Bact. suipestifer, Proteus, Bact. pyocyaneus* and of all of the *Bact. dysenteriae* group, none died within a month. Of the seven strains of

Bact. coli used, however, three strains died within five, eight and ten days, while the four other strains remained alive until after one month.

Fate of colon typhoid group on different sugar cultures, inoculated at 37°C. Numerals signify day of death. Mark – still alive thirty days afterward

ORGANISM	SUGAR CULTURES					
	Glucose peptone		Mannitol peptone		Lactose peptone	Sucrose peptone
	Results for all strains	Average number of days	Results for all strains	Average number of days	All strains	All strains
1. *Bact. typhi*	3 to 7	3 to 5	4 to 10	4 to 6	–	–
2. *Bact. paratyphi* A	3 to 7	3 to 5	4 to 8	4 to 5	–	–
3. *Bact. paratyphi* B	3 to 10	5 to 6	4 to 10	5 to 8	–	–
4. *Bact. enteritidis*	5 to 7	5 to 7	7 to 9	8	–	–
5. *Bact. suipestifer*	5 to 8	7	7 to 9	8	–	–
6. *Bact. coli*	5 to 8	6 to 7	5 to 16	5 to 8	5 to 18	3 strains, 5, 8, and 10 days; 4 strains, –
7. Proteus	6 to 9	7	–		–	–
8. *Bact. pyocyaneus*	–		–		–	–
9. Dysentery						
a. Shiga no. 1	3		10		–	–
b. Shiga no. 2	5		–		–	–
c. Flexner no. 1	6		10		–	–
d. Flexner no. 2	7		7		–	–
e. Hiss	7		7		–	–
f. Strong	5		5		–	–

In the above experiments we used 1 or 3 per cent sugar, but the results were almost the same with both concentrations.

From the results of these experiments we conclude that the recording of sugar fermentation reaction after the organisms are dead is unneceessary. It is desired to observe their activity only during their life. However, we still noticed more or less difference in the amount of the acid or alkali, this variation depending upon the absorption of CO_2 from the atmosphere.

For instance, in the glucose culture medium for the *Bact. typhi* and *Bact. paratyphi* group three to four days will be enough to take the record, then the tubes may be discarded; for *Bact.*

enteritidis, *Bact. suipestifer*, or *Bact. dysenteriae* group the period depends upon the strains, but about five to seven days will be enough; for *Bact. coli* six to seven days suffice; in regard to the other bacilli and also the other sugar media reference can be made to the accompanying table; the number of days indicated to study the reaction of the sugar cultures with the colon-typhoid group are sufficient.

"COLOR STANDARDS" FOR THE COLORIMETRIC MEASUREMENT OF H-ION CONCENTRATION pH 1.2 TO pH 9.8

LEON S. MEDALIA

From the Research Laboratories, Department of Biology and Public Health, Massachusetts Institute of Technology

Received for publication April 7, 1920

I. H-ION CONCENTRATION METHOD OF ADJUSTING CULTURE MEDIA BY THE USE OF "COLOR STANDARDS"

Introduction

Sörensen in 1909 demonstrated the value of the "H-ion concentration" as a basis for measuring the reaction in organic fluids as against the "titration" method then in vogue for adjusting culture media. This method has been adopted by practically all research workers in bacteriology ever since the classic work of Sörensen was published, yet no standard text-book in bacteriology however recent, does more than make a passing reference to it, although they all agree upon the value of the proper titration of culture media. It occurred to the writer that the reason why this method of determining reaction was left to the research worker alone, was because of the difficulties encountered in its application. In other words to carry out the standardization of culture media, according to the H-ion concentration properly, as heretofore suggested, one needs to be a fairly well trained chemist as well as a mathematician of no mean degree. To overcome these difficulties and make this very valuable method of adjusting culture media adaptable to the average laboratory, this research was undertaken. Only the successful results and method will be given in this report, all the attempts and experiments that led up to these results being omitted. Expressions that require a knowledge of higher mathematics and chemistry will be

strictly avoided in order not to confuse the average laboratory worker. Those interested in the subject, will find the articles by Sörensen (1912), and Clark and Lubs (1917), of special value.

Principles underlying the H-ion concentration method of titration

This method is based upon the *electrolytic dissociation* theory or *ionic-theory* developed by Arrhenius in 1887, which assumes that acids, bases, or salts, in aqueous solutions are dissociated to a greater or less extent into *ions*, i.e., electrically charged atoms, or groups of atoms. The unit charge is that which is associated with one hydrogen-ion. The strength of an acid in solution, according to this theory, depends upon the free or dissociated hydrogen-*ions* present in that solution. The comparative strength of two equivalent or "normal" solutions will vary according to the ability of the components of each to dissociate and allow free hydrogen-*ions* to accumulate in the solution. The relative amount of free hydrogen-*ions* (percentage dissociation) in "normal" solutions has been found to show wide variations. Thus, the percentage dissociation or the amount of H-ions set free in $\frac{N}{10}$ HCl solution was found (Talbot 1908) to be 90 per cent, while that of $\frac{N}{10}$ acetic acid is 1.4 per cent. Hydrochloric acid according to these findings is therefore 64 times stronger in acidity than acetic acid, although 10 cc. of $\frac{N}{10}$ of either acid will require a similar 10 cc. portion of $\frac{N}{10}$ NaOH to neutralize it. Accordingly the only correct method of measuring the acid strength of a solution is to determine the amount of free H-ions or the *hydrogen-ion concentration* (H.I.C.), of that solution, and *not* to determine the amount of $\frac{N}{10}$ NaOH necessary to neutralize that solution.

Another grave source of error in the titration method in common vogue is the fact that organic substances known as "buffers" when present in solutions (such as culture media) are capable of combining with the $\frac{N}{10}$ NaOH added during the titration, deviating it from and preventing its neutralizing, the acid with which it was meant to react.

From the foregoing it is evident that the NaOH method of titration with phenolphthalein is erroneous and highly misleading.

Method of expressing H-ion concentration—what is pH?

The accumulation of free hydrogen-ions present in a given solution, i.e., the H. I. C. of that solution, can be measured to the minutest amount and has been expressed in terms of "normal solutions." The amounts are so minute that they run up to the billionth or trillionth normal, since the acid strength or the hydrogen-ion content of neutral or even alkaline solutions

TABLE 1

Comparative values of hydrogen ion concentration expressed in "pH" and "normal" solutions

	pH	NORMAL SOLUTION (1 GRAM H TO 1 LITER OF WATER)	MICRONORMAL (μN.) SOLUTION*
$\frac{N}{1}$ HCl	0.0	1.0	1,000,000.0
$\frac{N}{10}$ HCl	1.0	0.1	100,000.0
	2.0	0.01	10,000.0
	3.0	0.001	1,000.0
	4.0	0.000,1	100.0
	5.0	0.000,01	10.0
	6.0	0.000,001	1.0
Neutrality	7.0	0.000,000,1	0.1
Alkaline	8.0	0.000,000,01	0.01
	9.0	0.000,000,001	0.001
	10.0	0.000,000,000,1	0.000,1
	11.0	0.000,000,000,01	0.000,01
	12.0	0.000,000,000,001	0.000,001
$\frac{N}{10}$ NaOH	13.0	0.000,000,000,000,1	0.000,000,1
$\frac{N}{1}$ NaOH	14.0	0.000,000,000,000,01	0.000,000,01

* By Micronormal is meant one-millionth (1/1,000,000) normal. Symbol μN.

is measurable. In order to overcome the unwieldiness of the figures necessary to express the H. I. C. Sörensen suggested the symbol pH to express one-tenth normal beginning on the acid side and going up in negative multiples of one-tenth towards alkalinity. Thus pH 1 equals $\frac{N}{10}$ acid; pH 2 equals $\frac{N}{10} \times \frac{N}{10} = \frac{N}{100}$; pH 3 equals $\frac{N}{100} \times \frac{N}{10} = 1/1000$ normal, etc. The lower the pH of a given solution, therefore, the more acid, or the higher its H. I. C. and the higher the pH the less acid, or the lower is its

TABLE 2

*Comparative values of H. I. C. expressed in pH, with fractions and normal solutions**

pH	NORMAL	MICRONORMAL (μN)
0.0	1.0	1,000,000.0
1.0	0.1	100,000.0
1.1	0.079,43	79,430.0
1.2	0.063,10	63,100.0
1.3	0.050,12	50,120.0
1.4	0.039,81	39,810.0
1.5	0.031,62	31,620.0
1.6	0.025,12	25,120.0
1.7	0.019,95	19,950.0
1.8	0.015,85	15,850.0
1.9	0.012,59	12,590.0
2.0	0.01	10,000.0
2.1	0.007,943	7,943.0
2.2	0.006,310	6,310.0
2.3	0.005,012	5,012.0
2.4	0.003,981	3,981.0
2.5	0.003,162	3,162.0
2.6	0.002,512	2,512.0
2.7	0.001,995	1,995.0
2.8	0.001,585	1,585.0
2.9	0.001,259	1,259.0
3.0	0.001	1,000.0
3.1	0.000,794,3	794.3
3.2	0.000,631,0	631.0
etc.		
4.0	0.000,1	100.0
4.1	0.000,079,43	79.43
4.2	0.000,063,10	63.10
etc.		
5.0	0.000,01	10.0
5.1	0.000,007,943	7.943
5.2	0.000,006,310	6.310
etc.		
6.0	0.000,001	1.0
6.1	0.000,000,794,3	0.794,3
6.2	0.000,000,631,0	0.631,0
etc.		

* This table was prepared from an antilogarithm table by using the antilogarithmic value of 0.9 for pH 1.1, that of 0.8 for pH 1.2, etc.—since pH 1.1 is pH 0.9 stronger than pH 2, and pH 1.2 is pH 0.8 stronger than pH 2, etc.

TABLE 2—*Continued*

pH	NORMAL	MICRONORMAL (μN)
7.0	0.000,000,1	0.1
7.1	0.000,000,079,43	0.079,43
7.2	0.000,000,063,10	0.063,10
etc.		
8.0	0.000,000,01	0.01
8.1	0.000,000,007,943	0.007,943
8.2	0.000,000,006,310	0.006,310
etc.		
9.0	0.000,000,001	0.001
9.1	0.000,000,000,794,3	0.000,794,3
9.2	0.000,000,000,631,0	0.000,631,0
etc.		
10.0	0.000,000,000,1	0.000,1

H. I. C. The relationships of pH to normal are given in tables 1 and 2. Table 1 gives the comparative values of pH and normal solutions, and micronormal solutions. (The term micronormal meaning a millionth normal, symbol (μ.N.) was coined by the writer to facilitate expressing pH values in terms of normal solution.) Table 2 gives the values of pH and its decimal fractions in terms of normality. These tables were thought desirable because we are in a habit of thinking in terms of normality, and not in terms of pH.

Methods used to determine the H-ion concentration

Having decided upon the H-ion concentration as a correct method to determine the acidity of a given solution the question arises how this may be accomplished? Two methods have been described (1) the electrolytic method, and, (2) the colorimetric method.

The electrolytic method, though the more accurate, is highly technical and requires cumbersome apparatus much beyond the possibilities of the average bacteriological laboratory.

The colorimetric method depends on the *color changes* that take place in certain indicators at different acid strength or H-ion concentration. The H-ion concentration has been determined electrolytically for a number of solutions ("buffer solutions") con-

taining various chemicals in different dilutions. Such solutions when accurately duplicated are supposed to be of the same H-ion content, and should therefore show similar variations in color, in the indicators used. These solutions are referred to as "standard solutions" of known H. I. C.

The difficulties met with in preparing the "standard solutions" of known H. I. C. in the average bacteriological laboratory constitute one reason why this method has not come into general use, and furnished the principal stimulus for this research.

Experimental work

In looking over the range of pH of the indicators developed by Clark and Lubs (1917) I found that the sensitive range between the extreme acid color and the extreme alkaline color of each indicator, is pH 1.6. It occurred to me, that by dividing this sensitive range of color, pH 1.6, into *eight equal* parts, one should obtain a range for each indicator of pH 0.2 intervals. This should be accomplished by adding 0.1 cc. of the indicator solution to one test tube containing 10 cc. of an alkaline solution, and 0.7 cc. of the same indicator to another test tube containing 10 cc. of an acid solution—(solutions that will bring out the alkaline and acid colors of the particular indicator)—then looking through the two test tubes placed one behind the other, i.e., superimposing the alkaline and acid colors of the indicator in different strengths[1] the color should be pH 0.2 higher (more alkaline) than the extreme acid color with 0.8 cc. of the same indicator. Increasing by 0.1 cc. in the alkaline solution, and decreasing by 0.1 cc. in the acid solution, in a set of seven pairs of tubes should give us the full range of the indicator at an interval of pH 0.2.

This was tested out with brom thymol blue and it succeeded perfectly, i.e., the green color was found at (pair no. 4) pH 7; or slightly yellowish green at (pair no. 3) pH 6.8, according to this range. (The change of color of this indicator was found by the

[1] Following the procedure made use of by Salm (1904) to determine the half transformation point and that devised by Barnett and Chapman to prepare the color standards for phenol red.

writer to start with pH 6.2 instead of pH 6 as given by Clark and Lubs (1917). Various difficulties had to be overcome in finding the proper acid or alkaline solution that would bring out the extreme colors that would remain fairly permanent. Finally, however, these difficulties were overcome and the proper strength of the indicators was found, that would best serve this purpose.

A method of obtaining the indicators in sterile solutions so that they could be kept indefinitely, and used for testing acid formation by bacteria in cultures without contaminating them was also devised.

Preparation of indicator solutions

a. Stock alcoholic solutions. Two-tenths per cent alcoholic solutions of the indicators were prepared as "stock solutions" by dissolving 0.1 gram of the respective indicators in powder form in 50 cc. of 95 per cent ethyl alcohol (ordinary alcohol), and kept in amber colored bottles, well stoppered with rubber stoppers and paraffined. The following indicators of Clark and Lubs were used, all of which were obtained in powder form from Hynson Westcott and Dunning excepting methyl red which was obtained at the Institute. Range and color changes are as given by Clark and Lubs (1917).

Indicators (Clark and Lubs)

1. Thymol blue acid range	Red-yellow	pH 1.2–2.8
2. Brom phenol blue	Yellow-blue	pH 3.0–4.6
3. Methyl red	Red-yellow	pH 4.4–6.0
4. Brom cresol purple	Yellow-purple	pH 5.2–6.8
*5. Brom thymol blue	Yellow-blue	pH 6.0–7.6*
6. Phenol red	Yellow-red	pH 6.8–8.4
7. Cresol red	Yellow-red	pH 7.2–8.8
8. Thymol blue alkaline range	Yellow-blue	pH 8.0–9.6

* The range of color of this indicator as found by the writer should read 6.2–7.8.

These "stock" alcoholic solutions are best kept in a dark place —closed cupboard, or box.

b. Indicator watery solutions. The strength of the indicator solutions found most useful to prepare the "standard colors" as well as for use in measuring the H. I. C. of fluids were 0.02 per cent of the respective indicators in sterile distilled water, (excepting phenol red in which case 0.04 per cent was found necessary). These solutions were prepared from the "stock alcoholic solutions" by placing 45 cc. (40 cc. for phenol red) of distilled water in each of eight amber colored bottles of proper size stoppered with cotton and sterilized in the autoclave at 10 pounds for one-half hour (rubber stoppers were sterilized alongside). To each of the 45 cc. bottles of sterile distilled water, 5 cc. of the "stock" alcoholic solutions of the indicators were added with sterile pipets in an aseptic way making a 0.02 per cent solution, except in the case of phenol red, in which case 10 cc. of the alcoholic stock solutions were added (to the 40 cc. of water) making a 0.04 per cent solution. These water solutions were kept in the dark, well stoppered with the sterilized rubber stoppers.

The flocculent precipitate of the methyl red, formed when the alcoholic solution was added to the distilled water, was cleared away by the addition of 0.5 cc. of sterile $\frac{N}{10}$ NaOH. This has not interfered with the production of red in the acid, and yellow in the alkaline solutions.

Preparation of "color standards"

For the preparation of "color standards" for standardizing culture media, the regular B. B. H. test tubes were used (thick walled, without lips, 130 by 16 mm. outside diameter) 14 or at most 28 such tubes being necessary. They should be of clear glass and as *nearly alike* in *diameter* as possible. The importance of having the tubes alike in diameter cannot be emphasized too strongly.

Two sets of test tubes, seven pairs in each, are made use of, one set for *brom thymol blue*, and the other for *phenol red*. Seven tubes of each set are filled with approximately $\frac{N}{10}$ NaOH, and the other seven with 0.1 per cent HCl (made from concentrated HCl 0.1 cc. and distilled water 100 cc.). The seven pairs of tubes are

set up in a rack, the tubes containing the acid solution in a row, behind which is another row of seven tubes containing the alkaline solution (fig. 1). Brom thymol blue, 0.02 per cent watery solution, is added to the tubes with NaOH in amounts of 0.1 cc. increasing by 0.1 cc. up to 0.7 cc. from left to right, while in the acid tubes the indicator is added beginning with 0.7 cc. decreasing by 0.1 cc. to 0.1 cc. from left to right, respectively: so that each pair of tubes contains together 0.8 cc. of the indicator, the amount increasing by 0.1 cc. in the tubes with the alkaline solution, while it decreases by 0.1 cc. in the acid solution. The tubes are then labelled in pairs from 1 to 7, the label also bearing the name of the indicator and the pH. The pH of the first pair is always 0.2 higher (more alkaline) than the initial range of the particular indicator. Brom thymol blue, according to my findings, begins to show a change of color at pH 6.2 (no difference in color was found with this indicator between pH 6, and 6.2, but there is a difference between 6.2 and 6.4). The first pair of this indicator will accordingly be pH 6.4, each succeeding pair will increase by pH 0.2 up to the seventh pair which will be pH 7.6. Therefore the seven pairs with brom thymol blue yield a range of "standard colors" of pH 6.4 to pH 7.6, at an interval of pH 0.2, most valuable for titration of culture media since neutrality is at pH 7, which with this indicator is at pair no. 4 yielding a green color—not yellowish green (pair no. 3) nor bluish green (pair no. 5). If a wider range is desired (rarely necessary) a set of "standard colors" with the phenol red can be prepared in the same way as given for brom thymol blue, i.e., the same strength of NaOH and HCl is used, the only difference being the change of the indicator to phenol red. The seven pairs of tubes of this indicator will yield a range from pH 7 to pH 8.2 overlapping at pH 7, 7.2, 7.4, and 7.6. The phenol red does not make as clear cut a range, as easily differentiated at pH 0.2 intervals, as does brom thymol blue and is not really necessary for the titration of culture media which as a rule are neutral, or thereabouts. Figure 1 shows the way the test tubes are set up for the preparation of the "color standards." Tables 3 and 4 give the composition of each pair for the two indicators, the color change of each, as nearly as it can be described, and the pH value of each pair.

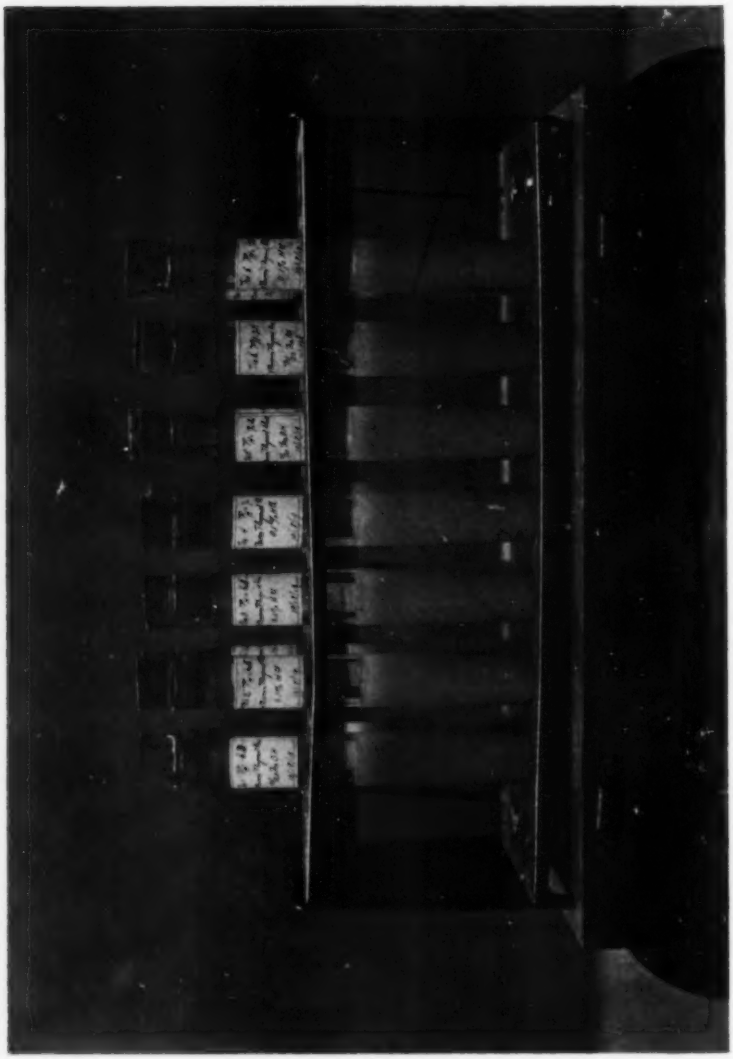


FIG. 1. BLACK ENAMELLED IRON TEST TUBE SUPPORT

Single deck with double row of holes containing seven pairs of "color standards" of brom thymol blue. (The second tube of pair no. 6 was placed in front of pair no. 7 by mistake, it should read no. 7 pH 7.6.)

The pH values have been calibrated with solutions of known H. I. C. prepared according to directions given by Clark and Lubs (1917) and cross checked where the indicators overlap.

Keeping qualities of the "standard colors." The standard color solutions have been kept for three months by observing the following rules in their preparation. New test tubes were washed in *warm water* (hot water produced a coating on the inside of some of the tubes) dried and filled with the respective $\frac{N}{20}$ NaOH and 0.1 per cent HCl freshly prepared with fresh distilled water. The tubes were then set up and the indicator solutions added, as

TABLE 3

*Composition of "Standard Colors" prepared with brom thymol blue 0.02 per cent watery solution—range pH 6.4 to 7.6**

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 per cent HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	6.4
2	1	10	0.2	2	10	0.6	Lighter yellow	6.6
3	1	10	0.3	2	10	0.5	Yellow-green	6.8
4	1	10	0.4	2	10	0.4	Green	7.0
5	1	10	0.5	2	10	0.3	Bluish green	7.2
6	1	10	0.6	2	10	0.2	Greenish blue	7.4
7	1	10	0.7	2	10	0.1	Blue	7.6

* The useful range of color of this indicator, according to calibration with phosphate NaOH mixture, should begin with pH 6.2, instead of pH 6, as given by Clark and Lubs.

TABLE 4

Composition of "standard colors" prepared with phenol red 0.04 per cent watery solution—range pH 7 to pH 8.2

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 per cent HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*			
1	1	10	0.1	2	10	0.7	Yellow	7.0
2	1	10	0.2	2	10	0.6	Lighter yellow	7.2
3	1	10	0.3	2	10	0.5	Pink yellow	7.4
4	1	10	0.4	2	10	0.4	More pink yellow	7.6
5	1	10	0.5	2	10	0.3	Beginning red	7.8
6	1	10	0.6	2	10	0.2	Slightly more red	8.0
7	1	10	0.7	2	10	0.1	Red	8.2

described, with pipettes. The tubes were then stoppered with corks previously dipped in hot melted paraffin. This procedure yields tightly sealed paraffined tubes of "standard color" solutions.

Titration of culture media

For the titration of a culture medium[2] three test tubes, similar to those used for the "color standards," are filled, each with 2 cc. of the filtered medium and 8 cc. of distilled water. To one of these 0.8 cc. of the 0.02 per cent of the brom thymol blue indicator is added and compared with the "color standards" tubes. The other two tubes are used to offset the color of the medium in the "comparator" block described below. The fluid to be titrated is placed in the center row of the "comparator" block having two test tubes of distilled water behind it. On either side are placed the two pairs of test tubes nearest to the pH desired (pairs 4 and 5 pH 7 and pH 7.2). In front of each pair is placed the tube containing 2 cc. of the medium and 8 cc. of distilled water to offset the color of the medium. It is absolutely *necessary* to *compensate* for the color of the medium in this way or the result will be misleading. $\frac{N}{20}$ NaOH is carefully run in the tube that is being titrated until it matches pair no. 4 = pH 7. Twenty-five times the amount of $\frac{N}{20}$ NaOH used, will represent the amount of normal NaOH necessary to neutralize 1 liter of the medium.

$$\left(\frac{1000 \text{ cc.}}{2 \text{ cc.}} \times \frac{1}{20} = 25\right)$$

If medium is too alkaline add $\frac{N}{20}$ HCl until the color matches and calculate the amount of $\frac{N}{1}$ HCl to be added to one liter of medium in the same way. The same factor is used whatever the pH value selected for the reaction of the medium. The medium should be retitrated after the addition of the alkali or the acid as the case may be. The use of the "comparator block"

[2] Hot fluid agar or gelatin media, before final filtration, can be titrated as described. The 2 cc. of the agar or gelatin when diluted with 8 cc. of water, remain fluid for a much longer time than is necessary to carry out the titration.

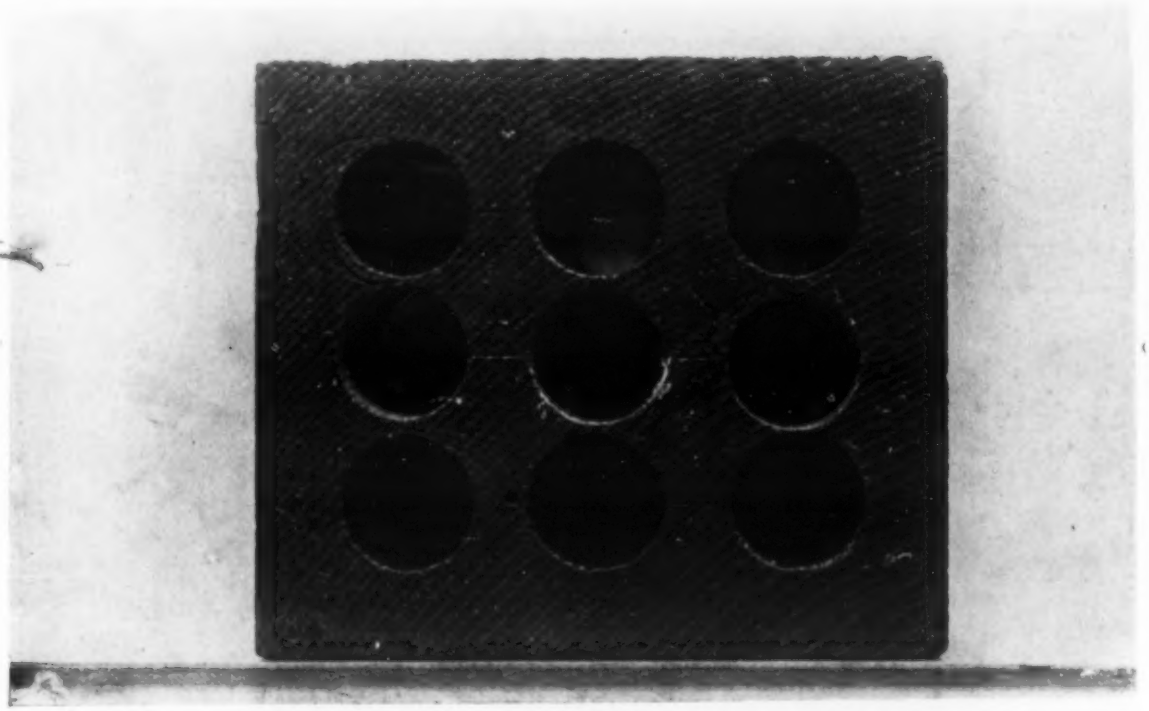


FIG. 2. TOP OF "COMPARATOR BLOCK," NATURAL SIZE—SHOWING THE THREE ROWS OF HOLES

In the front, center hole, is placed the tube containing the culture media to be titrated, behind it are placed two tubes of distilled water—on either side are placed the pairs of "color standards" desired with a tube of culture media behind each pair to offset the color of the medium.

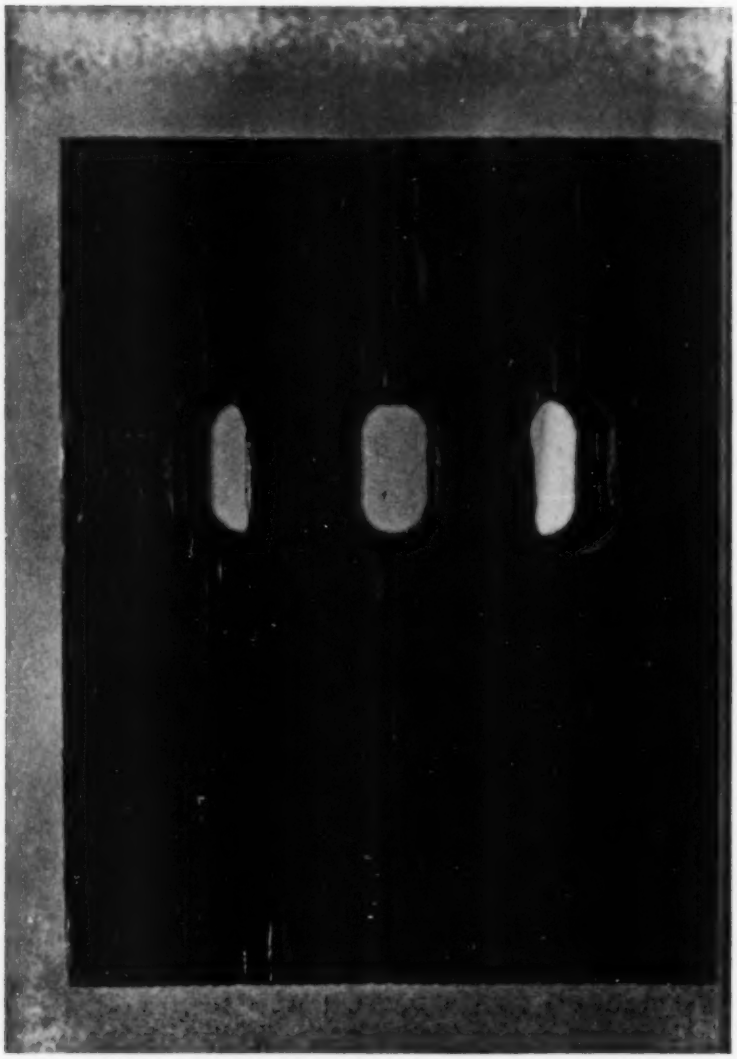


FIG. 3. FRONT OF "COMPARATOR BLOCK," NATURAL SIZE

Showing the three slits connecting the three rows of holes holding the test tubes. The slits are used to look through the test tubes as they are placed one behind the other.

such as described by Hurwitz, Myer and Ostenberg (1916) modified to suit our purpose, was found indispensable.

The block is made by boring three rows of three holes in each row. The holes are 2 cm. in diameter and 9 cm. deep. The block measures 13 cm. high by 9 by 10 cm. Each row of holes is connected by a slit that goes all the way through, 2.5 cm. high by 1.25 cm. wide, the upper edge of the slit being 4 cm. from top of block. The block is painted with black enamel paint including the slits. It is best not to paint the holes that hold the test tubes (figs. 2 and 3).

II. pH MEASUREMENT OF ACID OR ALKALI PRODUCTION BY BACTERIA, BY THE USE OF "COLOR STANDARDS"

A wider range is necessary to measure the acid and alkali production by bacteria than that used for the standardization of culture media. "Color standards" prepared from 0.02 per cent solutions of each of the following indicators: Thymol-blue-acid-range; brom-phenol-blue; methyl-red; brom-cresol-purple; cresol-red; and thymol-blue-alkaline-range; in addition to brom-thymol-blue, and phenol-red, used for titration of culture media, already described, will give a range extending from pH 1.4 to pH 9.6 more than sufficient to test the acid as well as the alkali production by bacteria.

The methods for the preparation of "color standards" from these additional indicators are similar to those described in part I. The indicators are used in 0.02 per cent watery solutions prepared from 0.2 per cent stock alcoholic solutions (phenol-red 0.04 per cent).

The solutions necessary to bring out the extreme acid color, and the extreme alkaline color of the various indicators are as follows.

Thymol-blue acid range. The *extreme acid* (red) color of this indicator was brought out by 0.5 per cent HCl; and the *extreme alkaline* (yellow) of this indicator was brought out with 0.001 per cent HCl (1 cc. of 0.1 per cent to 99 cc. of distilled water). Various other strengths of both acid and alkaline solutions were tried, but failed.

Thymol-blue-alkaline-range. The extreme acid (yellow) color was brought out by using 0.001 per cent HCl; the extreme alkaline (blue) color of this indicator was brought out by the use of $\frac{N}{20}$ NaOH.

Brom-phenol-blue. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (blue) color was brought out by $\frac{N}{200}$ NaOH solution, instead of $\frac{N}{20}$ NaOH.

Methyl-red. The extreme acid (red) color of this indicator, was brought out by 0.1 per cent HCl; the extreme alkaline (yellow) color of this indicator was brought out by $\frac{N}{20}$ NaOH.

Brom-cresol-purple. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (purple) color was brought out by $\frac{N}{20}$ NaOH.

Brom-thymol-blue. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (blue) color was brought out by $\frac{N}{20}$ NaOH.

Phenol-red. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (red) color was brought out by $\frac{N}{20}$ NaOH.

Cresol-red. The extreme acid (yellow) color of this indicator was brought out by 0.1 per cent HCl; the extreme alkaline (red) color was brought out by $\frac{N}{20}$ NaOH.

The procedure is the same as described under part I—seven pairs of tubes are filled, each with 10 cc. amounts of the respective acid and alkaline solutions for each indicator. The seven alkaline (or weak acid) tubes are set up in a row behind the seven acid tubes. The indicator solution is added to the akaline tubes in amount of 0.1 cc. *increasing* by 0.1 cc. up to 0.7 cc. from left to right—while in the acid row of tubes, 0.7 cc. indicator solution is added *decreasing* from left to right by 0.1 cc. down to 0.1 cc. in the last tube. In case it is desired to keep the color standards for any length of time, it is best to stopper the tubes with cork stoppers previously dipped in hot melted paraffin. Solutions treated as just described, have been found to keep well for over three months. It is well to check up these "standard colors" by setting up fresh sets, for the purpose, once a month, or oftener. This can be done very easily and does not take up much time.

TABLE 5

Composition of "standard colors" prepared with thymol-blue-acid—range 0.02 per cent watery solution—range pH 1.4 to pH 2.6

PAIR	TUBE	0.001 PER CENT HCl	INDICATOR	TUBE	0.5 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Red	1.4
2	1	10	0.2	2	10	0.6	Lighter red	1.6
3	1	10	0.3	2	10	0.5	Yellow red	1.8
4	1	10	0.4	2	10	0.4	Red yellow	2.0
5	1	10	0.5	2	10	0.3	Beginning yellow	2.2
6	1	10	0.6	2	10	0.2	More yellow	2.4
7	1	10	0.7	2	10	0.1	Still more yellow	2.6

TABLE 6

Composition of "standard colors" prepared with brom-phenol-blue 0.02 per cent watery solution—range pH 3.4 to pH 4.6*

PAIR	TUBE	$\frac{N}{200}$ NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	3.4
2	1	10	0.2	2	10	0.6	Light yellow	3.6
3	1	10	0.3	2	10	0.5	Beginning pink	3.8
4	1	10	0.4	2	10	0.4	Pink-red	4.0
5	1	10	0.5	2	10	0.3	More red	4.2
6	1	10	0.6	2	10	0.2	Beginning blue	4.4
7	1	10	0.7	2	10	0.1	Blue	4.6

* The blue color of this indicator can be brought out with $\frac{N}{20}$ NaOH, but is not lasting and fades within twenty-four to forty-eight hours, while the $\frac{N}{200}$ NaOH makes a lasting range of "color standards."

TABLE 7

Composition of "standard colors" prepared with methyl red 0.02 per cent watery solution—range pH 4.6 to pH 5.8

PAIR	TUBE	$\frac{N}{20}$ NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Red	4.6*
2	1	10	0.2	2	10	0.6	Light red	4.8
3	1	10	0.3	2	10	0.5	Yellow red	5.0
4	1	10	0.4	2	10	0.4	Red yellow	5.2
5	1	10	0.5	2	10	0.3	Beginning yellow	5.4
6	1	10	0.6	2	10	0.2	More yellow	5.6
7	1	10	0.7	2	10	0.1	Yellow	5.8

* There was a very slight difference between the phthalate NaOH mixture and the "standard colors" in the first three pairs: Pair No. 1 = pH 4.7; Pair No. 2 = pH 4.9; Pair No. 3 = pH 5.1 according to the phthalate NaOH mixture, the rest were the same in both.

TABLE 8

Composition of "standard colors" prepared with brom-cresol-purple 0.02 per cent watery solution—range pH 5.4 to pH 6.6

PAIR	TUBE	N/20 NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	5.4
2	1	10	0.2	2	10	0.6	Lighter yellow	5.6
3	1	10	0.3	2	10	0.5	Beginning pink	5.8*
4	1	10	0.4	2	10	0.4	Pink	6.0
5	1	10	0.5	2	10	0.3	Beginning purple	6.2
6	1	10	0.6	2	10	0.2	More purple	6.4
7	1	10	0.7	2	10	0.1	Purple	6.6

* According to phthalate and phosphate NaOH mixtures, pairs 3, 4, 5, 6, 7, should read: pH 5.9; 6.1; 6.3; 6.5; 6.7.

TABLE 9*

Composition of "standard colors" prepared with cresol-red 0.02 per cent watery solution—range pH 7.4 to pH 8.6

PAIR	TUBE	N/20 NaOH	INDICATOR	TUBE	0.1 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	7.4
2	1	10	0.2	2	10	0.6	Lighter yellow	7.6
3	1	10	0.3	2	10	0.5	Pink yellow	7.8
4	1	10	0.4	2	10	0.4	Yellow pink	8.0
5	1	10	0.5	2	10	0.3	Pink	8.2
6	1	10	0.6	2	10	0.2	Beginning red	8.4
7	1	10	0.7	2	10	0.1	Red	8.6

* For the composition of "standard colors" prepared with *brom-thymol-blue* and *phenol-red* see table 3 and table 4.

TABLE 10

Composition of "standard colors" prepared with thymol-blue-alkaline-range 0.02 per cent water solution—range pH 8.2 to 9.4

PAIR	TUBE	N/20 NaOH	INDICATOR	TUBE	0.001 PER CENT HCl	INDICATOR	COLOR	pH
		cc.	*cc.*		*cc.*	*cc.*		
1	1	10	0.1	2	10	0.7	Yellow	8.2
2	1	10	0.2	2	10	0.6	Light yellow	8.4
3	1	10	0.3	2	10	0.5	Yellow-green	8.6
4	1	10	0.4	2	10	0.4	Green	8.8
5	1	10	0.5	2	10	0.3	Green-blue	9.0
6	1	10	0.6	2	10	0.2	Faint blue	9.2
7	1	10	0.7	2	10	0.1	Blue	9.4

The detailed composition of the pairs of tubes comprising the "color standards" of each indicator, as well as its equivalent in pH as found by calibration with standard solutions of known H. I. C. prepared according to Clark and Lubs (1917) will be found on tables 5, 6, 7, 8, 9, and 10; and part I, tables 3 and 4. The color of each pair of tubes corresponding to the particular pH is described as nearly as possible.

Procedure for measuring acid production by bacteria

In order to establish the best method to determine the acid production by bacteria quantitatively it was thought desirable to test out the various indicators as to their ability (a) to stand heating while being sterilized; (b) as to the action produced upon such indicators by bacteria. For that purpose the following experiments were undertaken:

Test tubes similar in size to those used for the "color standards" were filled each with 10 cc. amounts of glucose and of lactose bouillon (pH 7) using a 10 cc. automatic filler (fig. 4). These were divided into three sets of sixteen tubes each. To one set of the glucose and one set of the lactose bouillon 0.8 cc. amounts of the respective indicators were added to each tube *before* sterilizing. To another set, the same amount of the respective indicators was added *after* sterilizing, and the third set was used as a control—the indicators being added after inoculation with *Bact. coli* and incubation for twenty-four hours. Sterilization of the three sets was done at the same time by the fractional method in the Arnold sterilizer, on three successive days at 100°C. for three-quarters of an hour at a time.

Thus the *first* set was used to test the ability of the different indicators to stand heat. The *second* set was used to test the ability of the different indicators to resist the action of the bacteria; and the *third* set as a control of the first two sets. Table 11 gives the results of these tests in detail.

Action of heat upon the indicators used

A study of table 11 will show that after sterilization, seven of the indicators: thymol-blue-acid range; brom-phenol-blue; brom-cresol purple; brom-thymol-blue; phenol-red; cresol-red; and thymol-blue-alkaline range; did not change by sterilization in the

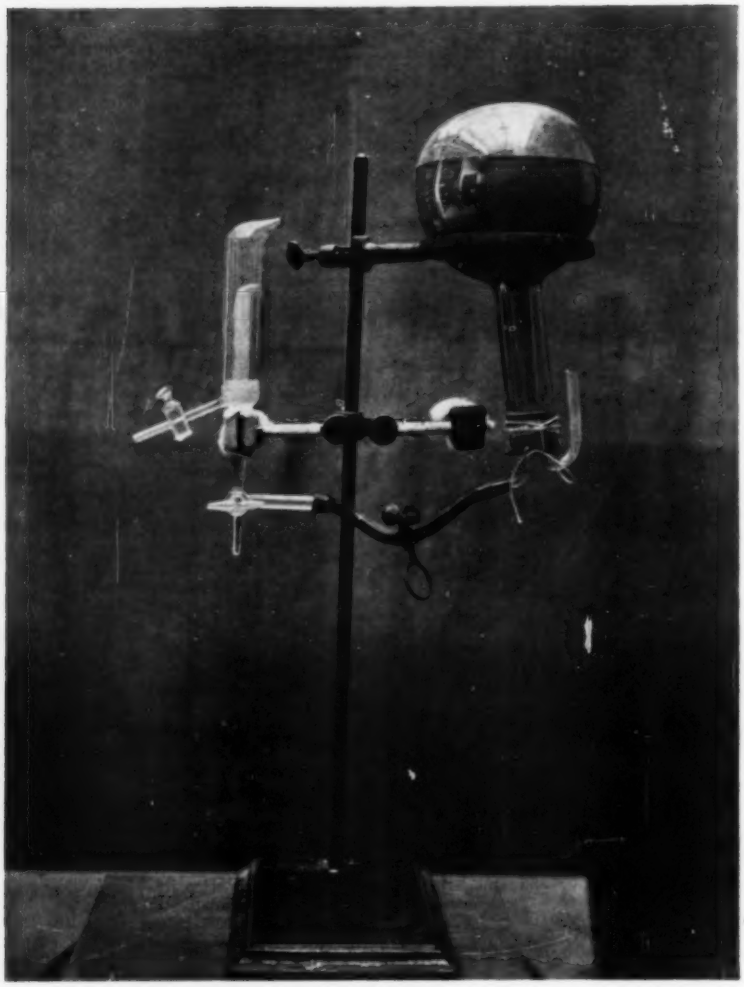


FIG. 4. AUTOMATIC 10 CC. FILLER

The flask of culture media is inverted and attached by a rubber tube to the 10 cc. automatic pipette (pipette Vanier) consisting of a three-way stopcock which allows the fluid to run into the inside tube which, when filled, measures exactly 10 cc. Any excess fluid flows over into a surrounding tube, which may be emptied by the side stopcock.

Arnold sterilizer. Methyl-red was the only indicator that decolorized on sterilization. This was true of the glucose as well as the lactose bouillon. These findings would justify the conclusion that the indicators enumerated, with the exception of methyl-red can be introduced in the sugar media before sterilization, if sterilization is done by the fractional method. It may be of interest to note here that the reaction of the glucose bouillon with brom-thymol-blue just before sterilization was pH 7, and after sterilization it was found to be pH 6.8; while the lactose bouillon was pH 7 before and the same after sterilization. This would tend to indicate that the glucose bouillon becomes slightly more acid on sterilization.

Action of bacteria upon the indicators

To each of the test tubes of the second set of glucose and lactose bouillon respectively, was added *after* sterilization and with sterile pipettes 0.8 cc. of each of the eight indicators enumerated. The third set was left as a control. All three sets were then placed in an incubator for twenty-four hours to test sterility. When found sterile, the next day they were inoculated with a pure culture of *Bact. coli* (freshly isolated from feces) and placed in an incubator at 37°C. According to the findings as given in table 11 none of the indicators except methyl red were attacked by the bacteria, (*Bact. coli*) used in this test. Methyl red was the only one of the whole group that was decolorized. It would seem fair, therefore, to assume that the seven indicators used may be added before sterilization and inoculation when dealing with *Bact. coli*. When dealing with another organism, it would be wise to test out its effect upon the indicators, or run control cultures to check up findings.[3]

[3] Incidentally it may be noted that the acid production of *Bact. coli* in glucose was found to be the difference between pH 6.8 and 4.8 or pH 2 in twenty-four hours, and that it did not increase in the next twenty-four hours The same organism in lactose produced much less acid in twenty-four hours (a difference of pH 7 to 5.5 or pH 1.5), while in the next twenty-four hours it reached the same acidity as in glucose (the difference of between pH 7 to 5 or pH 2). The third set used as a control, checked up the findings in set no. 1 and no. 2. Methyl-red if it be used, must be added fresh immediately before the reading is made. The ability of sulphonthalein indicators to withstand the action of bacteria, has been recognized by observers and referred to by the committee on descriptive chart. Com. Soc. Am. Bact. (1919).

TABLE 11

Data pertaining to the method of studying acid production by bacteria; also effect of heat and of bacteria (Bact. coli) upon the indicators used in glucose and lactose bouillon

	SET NO. 1. INDICATORS ADDED BEFORE STERILIZATION		SET NO. 2. INDICATORS ADDED AFTER STERILIZATION		SET NO. 3. INDICATORS ADDED AFTER INOCULATION AND INCUBATION	
	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose
Thymol-blue-acid-range						
After sterilization	No change (more yellow than pair no. 7)	No change in color	No change	No change		
Twenty-four hours incubation, *Bact. coli*	No change	No change	No change	No change	Same as sets 1 and 2	Same as sets 1 and 2
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change
Brom-phenol-blue						
After sterilization	No change in color	No change	No change	No change		
Twenty-four hours incubation, *Bact. coli*	Almost same as pair no. 7 pH 4.6	More blue than pair no. 7 (more alkaline than pH 4.6)	Same as glucose set no. 1	Same as lactose set no. 1	Same as glucose set no. 1	Same as lactose set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

			Methyl red			
After sterilization	Decolorized	Decolorized	More yellow than pair no. 7	Same as glucose		
Twenty-four hours incubation, *Bact. coli*	Decolorized	Decolorized	Decolorized	Decolorized	More red than pair no. 1 (more acid than pH 4.7)	Between pair nos. 4 and 5 (pH 5.3–5.4)
Forty-eight hours incubation, *Bact. coli*	Fresh indicator added—pH 4.7 (matches no. 1)	Fresh indicator added—pH 4.9 (matches no. 2)	Fresh indicator added—pH 4.7 (same as set no. 1)	Fresh indicator added—pH 4.9 (same as set no. 4)	Fresh 48 hour culture with fresh indicator—pH 4.7	Fresh 48 hour culture with fresh indicator—pH 4.9
			Brom cresol purple			
After sterilization	Changed to match pair no. 7 pH 6.7	Slightly more purple than pair no. 7 (more alkaline than pH 6.7)	Same as pair no. 7 pH 6.7	Slightly more purple than pair no. 7		
Twenty-four hours incubation, *Bact. coli*	More yellow than pair no. 1 (more acid than pH 5.4)	Same as dextrose	Same as set no. 1	Same as set no. 1	Same as set no. 1	Same as set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

TABLE II—*Continued*

	SET NO. 1. INDICATORS ADDED BEFORE STERILIZATION		SET NO. 2. INDICATORS ADDED AFTER STERILIZATION		SET NO. 3. INDICATORS ADDED AFTER INOCULATION AND INCUBATION	
	Glucose	Lactose	Glucose	Lactose	Glucose	Lactose
Brom thymol blue						
After sterilization	Matches pair no. 3 pH 6.8	Matches pair no. 4 pH 7	Same as set no. 1	Same as set no. 1		
Twenty-four hours incubation, *Bact. coli*	More yellow than no. 1 (more acid than pH 6.4)	More yellow than No. 1	Same as set no. 1	Same as set no. 1	Same as set no. 1	Same as set no 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change
Phenol red						
After sterilization	Slightly more yellow than pair no. 1 pH 7	Same as pair no. 1 pH 7	Same as set no. 1	Same as set no. 1		
Twenty-four hours incubation, *Bact. coli*	More yellow than no. 1 (more acid than pH 6.4)	Same as glucose	Same as set no. 1	Same as set no. 1	Same as set no. 1	Same as set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

Cresol red

After sterilization	More yellow than no. 1 pH 7.4 (less alkaline)	More yellow than no. 1	More yellow than no. 1	More yellow than no. 1		
Twenty-four hours incubation, *Bact. coli*	No change in color	No change	No change	No change	No change	No change
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

Thymol blue alkaline range

After sterilization	No change (more yellow than pair no. 1)	No change (more yellow than pair no. 1)	Same as set no. 1	Same as set no. 1		
Twenty-four hours incubation, *Bact. coli*	No change in color	No change	No change	No change	Same color as set no. 1	Same color as set no. 1
Forty-eight hours incubation, *Bact. coli*	No change	No change	No change	No change	No change	No change

The comparator block was used in all the above studies.

Sterile glucose bouillon and lactose bouillon were used in the comparator to offset the color of the bouillon in the studies before inoculation. Twenty-four hours control cultures and forty-eight hours control cultures were used in the comparator to offset the color and turbidity when studying the respective cultures.

Procedure

In order to test the acid production of any given organism it is only necessary to cultivate the organism in 10 cc. amounts of fluid culture media of a known pH, that will best favor its growth. To each of the tubes of culture media is added 0.8 cc. of a 0.02 per cent solution of indicator—either phenol-blue or brom-cresol-purple—before sterilization, or if methyl-red has to be used the 0.8 cc. (of 0.02 per cent watery solution) of this indicator has to be added just before the reading is done, i.e., after sterilization, inoculation, and incubation. The cultures are then inoculated and incubated for as long a time as desired. Control cultures without indicator are also made and incubated alongside of those containing the indicator. The control cultures are used in the comparator block to offset the color and turbidity. The actual reading of the pH is done by placing the culture containing the indicator say brom-cresol-purple in the front of the center row of the comparator block, having two tubes of distilled water behind it while on either side of it are placed the pairs of "standard color" tubes of brom-cresol-purple approaching in color that of the culture with the same indicator; in front of each pair of standard colors in the comparator block one of the control cultures is placed to offset the color and turbidity of the medium. The reading is, then, simply a matter of matching colors, and takes very little time. Thus the actual acid production by any given organism can be easily tested from hour to hour, if desired, without disturbing the culture in any way, except in the case of methyl-red where fresh cultures are necessary for each reading —because of the fact that it is unstable and must be added fresh each time a reading is made.

Testing for alkali production by bacteria

The procedure for testing the alkali production by bacteria could be carried out in a similar way to that just described for the acid production by *Bact. coli*. The only difference is that the proper indicators (phenol-red; brom cresol-red; and thymol-blue-alkaline range) must be substituted for those just described;

otherwise the procedure is exactly the same. It is best to have two different cultures side by side, one for the acid production with its respective indicator (brom-cresol-purple), and another culture for the alkali production with its respective indicator (cresol-red) rather than to use the two indicators in the same culture. The acid and alkali production by the same organism can thus be easily read off without the possibility of the color of either indicator offsetting the other.

III. pH MEASUREMENT OF THE H. I. C. OF OTHER FLUIDS AND SOLUTIONS BY MEANS OF "COLOR STANDARDS"

For measuring the H-ion concentration of other fluids and solutions, acid or alkaline, such as urine, blood serum, etc., the same procedure described under parts I and II may be followed. The color standards and the methods for their preparation will be found described under parts I and II and in the accompanying tables. It may be desirable to describe in detail the testing of urine as a guide to the testing of other fluids.

Procedure

Three test tubes of the same calibre as those used for the "color standards" are each filled with 10 cc. amounts of the urine to be tested, previously filtered. Then 0.8 cc. of the 0.02 per cent solution of the *proper*[4] indicator is added to one of the three tubes with the urine. The test tube is rolled between the palms of the hands until well mixed. This tube of urine containing the indicator is placed in the center hole of the "comparator block" with two test tubes filled with distilled water behind it. The *proper* pairs of color standards are placed, each pair on either side of the urine to be tested, while in front of each pair of color standards, one of the tubes of urine without indicator is placed in the comparator block to offset the color of the urine. The pH represented by the pair of color standard tubes that matches in color that of the urine with the indicator is the pH of the urine.

[4] Some idea as to which is the proper indicator to use may be obtained by using litmus paper. If highly acid to litmus, brom-phenol-blue is used; if slightly acid, methyl-red or brom-cresol-purple is used. If alkaline, brom-thymol-blue or cresol-red should be used. The pH is thus very easily read off.

If highly colored fluids are to be titrated they may be diluted with equal parts or more of distilled water, since the addition of distilled water does not change the H. I. C. materially, if at all. A little experience will greatly facilitate the practical application of this method, with respect to choosing the proper indicator to use, and in estimating readily which pair of color standard tubes to use.

SUMMARY AND CONCLUSIONS

The colorimetric measurement of the H. I. C. by means of the "color standards" described is the simplest method yet published.

These color standards are easily prepared, lasting, and their use is readily applicable in the average bacteriological laboratory.

The practical application of the "color standards" for the titration of culture media, by the H-ion concentration method; also for the pH measurement of the acid or alkali production by bacteria, and for the pH measurement of other fluids as described in this paper, is simple and easily handled, and because of its simplicity, it promises to take a foremost place in the colorimetric measurement of the H. I. C. of any fluid as well as in bacteriology.

In concluding I wish to express my appreciation to Professor W. T. Sedgwick through whose kindness this work was made possible, and to Professor S. C. Prescott, and the other members of the Department of Biology and Public Health of the Institute, for their helpful assistance rendered me in this work.

REFERENCES

Barnett, G. D., and Chapman, H. S. 1918 Colorimetric determination of reaction of bacteriologic mediums and other fluids. Jour. Am. Med. Assn., **70**, 1062–1063.

Clark, W. M., and Lubs, H. A. 1917 The colorimetric determination of hydrogen-ion concentration and its applications in bacteriology. Jour. Bact., **2**, 109–136; 191–236.

Committee of American Bacteriologists Report 1919 Jour. Bact., 1919, **4**, 107–132.

Hurwitz, Meyer, and Ostenberg 1916 Bull. Johns Hopkins Hospital, 1916, **27**, 16.

Salm, E. 1904 Ztschr. Electrochem., **10**, 341.

Sörensen, S. P. L. 1909 Ztschr. Biochem., **21**, 131, 201.

Sörensen, S. P. L. 1912 Ergebn. Physiol., **12**, 393.

Talbot, H. P. 1908 Quantitative Chemical Analysis. Macmillan Co., New York. Pp. 157.

A STUDY OF THE ACTION OF EIGHT STRAINS OF *BACT. ABORTIVO-EQUINUS* ON CERTAIN OF THE CARBOHYDRATES[1]

C. P. FITCH AND W. A. BILLINGS

University Farm, St. Paul, Minnesota

Received for publication April 10, 1920

Abortion in mares is a disease quite widely distributed in the United States. It does not, however, cause as extensive losses, comparatively, as does abortion disease among the bovine species. It has been shown that *Bact. abortivo-equinus* is the causative organism of abortion in mares in the United States and the biology and pathogenesis of the germ have been carefully studied, among others, by Good and Corbett, Meyer and Boerner in this country, and Van Heelsbergen and De Jong, and Dassonville and Reviere in Europe. Abortions by the mares at the Experiment Station at University Farm, Minnesota, have been somewhat frequent. As a preliminary to a detailed study of this infection several strains of *Bact. abortivo-equinus* were obtained and a careful study of the cultural and pathogenic properties of the organisms was undertaken. It was noted that the actions of the different strains varied on the same carbohydrate, notably in the case of lactose. That is, some strains fermented this sugar with the production of gas while others failed to do so. It was thought advisable to make a somewhat detailed study of the action of this germ on the carbohydrates to learn if the fermentive action was constant in the different strains and to determine the cause of possible variations.

Good and Corbett came to the following conclusions as a result of their study of gas production by different strains of *Bact. abortivo-equinus.*

[1] Published with the approval of the Director as Paper No. 188, of the Journal Series of the Minnesota Agricultural Experiment Station.

The bubble, or the small amount of gas, encountered so often in our fermentation tests with *Bacillus abortivc-equinus* in lactose and saccharose broth is not of physical, but of chemical, origin.

Bacillus abortivo-equinus produced approximately 2 per cent gas in lactose in 80 per cent of 116 trials, and in saccharose slightly less than 2 per cent gas in 50 per cent of 56 trials.

Bacillus abortivo-equinus may or may not produce gas in 1 per cent lactose or saccharose broth, even varying in this respect in duplicate and triplicate tests.

Bacillus abortivo-equinus possesses as an original physiologic characteristic the ability, in most cases, to ferment lactose to a small extent, and also, in some cases, to ferment saccharose to a less extent. This characteristic in all probability has not as yet been accentuated by environment.

In these tests the four strains of *B. abortivo-equinus* produced the following average percentages of gas in the carbohydrates which were fermented: xylose 51 per cent; raffinose 39 per cent; arabinose 59 per cent; sorbite 82 per cent; dulcite 95 per cent; glucose 74 per cent; mannite 81 per cent.

De Jong as a result of his original observations states that the germ which he isolated from the uterine exudate of mares which aborted does not form gas in lactose but does in sucrose. In an inaugural dissertation published later Van Heelsbergen working in the same laboratory as De Jong makes the positive statement that *Bact. abortivo-equinus* does not ferment either lactose or sucrose.

Meyer and Boerner state that *B. abortivo-equinus* does not ferment either lactose or sucrose.

Mudge found that heating in streaming steam seems to hydrolyze lactose and maltose and by sterilizing by filtration this hydrolysis can be avoided.

The study here reported was begun three years ago but owing to the fact that we were unable to obtain certain of the carbohydrates the work was very much delayed. The media used were prepared from lean beef, following the ordinary method. The muscle sugar was removed by the use of *Bact. coli* and then tested to insure that it was sugar free. One per cent of the various carbohydrates was added to this sugar free infusion bouillon.

The inverted vial was used to determine gas production. A portion of the medium, in the case of each carbohydrate, was distributed in test tubes, and sterilized in the Arnold by steaming twenty minutes for each of three successive days. Another portion of the same carbohydrate medium was sterilized by filtration through a Berkfeld filter employing the apparatus shown in figure 1. It will be noted from the cut that the test tubes are

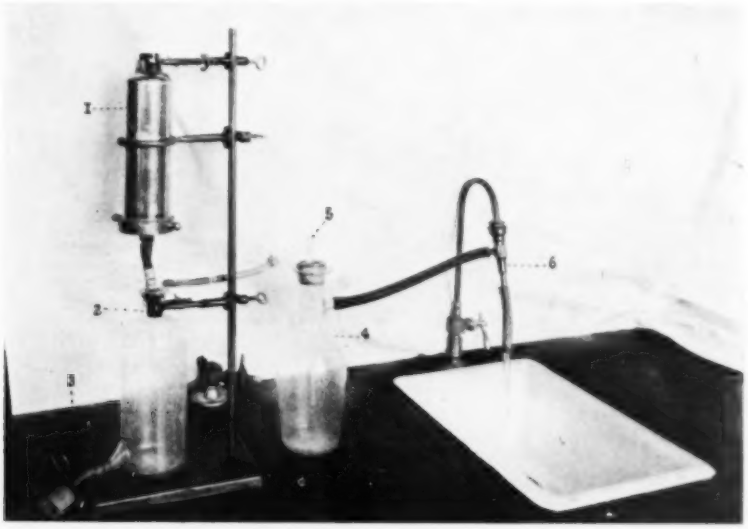


FIG. 1. APPARATUS SHOWING METHOD OF STERILIZATION OF MEDIA BY FILTRATION

1, Berkfeld filter case; *2*, receiver of filtered media; *3*, filling tube; *4*, wash bottle; *5*, air cock; *6*, water suction pump.

filled directly through the tube 3. The glass tube attached to the end prevents bacteria from dropping into the open test tube. The inverted vials were filled by first coating the cotton plugs of the tubes containing the medium with paraffin, allowing it to harden, and then inverting which would permit the medium to fill the vial. This unheated medium was incubated for forty-eight hours and then allowed to stand forty-eight hours at room temperature. The contaminated tubes were then removed.

The strains of *Bact. abortivo-equinus* were obtained as follows: GI, GII, and GIII, were sent us by Professor Good of Kentucky, M.7 and M.8 from the Bureau of Animal Industry, Washington, D. C., H.3 and H.13 from the Laboratory of the Pennsylvania Live Stock Sanitary Board, Philadelphia. Each strain was inoculated in a series of the heated and unheated media. A tube was left uninoculated as a check for each strain which was incubated with the others. In as much as the observations of Good and Meyer were made on the basis of the reaction of the culture,

Table showing the results of gas production by Bact. abortivo-equinus in heated and unheated carbohydrate media. Also a comparison with the results in heated media of other investigators

	HEATED MEDIA	UNHEATED MEDIA	RESULTS OF MEYER AND BOERNER	RESULTS OF GOOD AND CORBETT
Glucose	Gas	Gas	Gas	Gas
Lactose	Gas	No gas	No gas	80 per cent of trials
Sucrose	Gas*	No gas	No gas	50 per cent of trials
Maltose	Gas	Gas	Gas	Gas
Mannitol	Gas	Gas	Gas	Gas
Mannose	Gas	Gas	Gas	Not given
Levulose	Gas	Gas	Gas	Not given
Dulcitol	Gas	Gas	Gas	Gas
Raffinose	No gas	No gas	No gas	Gas
Arabinose	Gas	Gas	Gas	Gas
Rhamnose	Gas	Gas	Gas	No gas
Xylose	Gas	Gas	Gas	Gas
Melezitose	0 (Size of	bubble) 0	Not given	Not given
Inulin	Gas	Gas	No gas	Not given
Salicin	No gas	No gas	Not given	Not given

* None in one trial; very slight in another.

using phenolphthalein as an indicator, it was deemed advisable to continue with this system instead of employing the more accurate pH standard. The tables at the end of the article give the results of these observations, continued in nearly all cases over a period of thirty days. In some instances it was impossible to obtain at the time sufficient of the carbohydrate to complete the series. The figures give the amount of $\frac{N}{10}$ NaOH or $\frac{N}{10}$ HCl (if minus sign precedes) necessary to neutralize five mils of the culture.

The gas production is recorded as accurately as possible from the action in the inverted vials. Observations were made at twenty-four, forty-eight and seventy-two hour intervals. A comparison of the results in regard to gas production in the heated and unheated media is shown in the preceding table. The results of Meyer and Good, both of whom used heated media, are appended.

It will be noted that gas was obtained in lactose only from the heated media. Good and Corbett obtained gas in many trials with this sugar but Meyer and Boerner report "no gas." As a result of our work we believe that the gas production in lactose recorded by Good and Corbett is due to the hydrolysis of the carbohydrate by sterilization. The work of Mudge clearly pointed out that discontinuous Arnold sterilization hydrolizes lactose and the results here recorded show that no gas was produced by any of the strains in the unheated lactose media.

In sucrose bouillon a variation in the results obtained in the heated media is noted. In one trial no gas was produced by any strain, in another only a very small amount. None was found at any time in the unheated sucrose media. Good and Corbett likewise record a smaller number of positive gas productions in this carbohydrate while Meyer and Boerner record "no gas."

In this work all strains have failed to produce gas in raffinose in both the heated and unheated media while all strains have produced gas in rhamnose. The first trial (rhamnose) however, showed much less gas in the unheated media than the second. The fermentation of rhamnose with gas agrees with the work of Meyer and Boerner but is contrary to the results of Good and Corbett. A small amount of gas was formed by all strains in both heated and unheated inulin broth. In as much as *Bact. abortivo-equinus* produces large amounts of gas in maltose, the ease with which this sugar is broken down by heat sterilization is not shown.

The carbohydrates which are fermented with the production of large amounts of gas show a nearly steady increase in acidity during a thirty day period. Dulcitol is an exception to this rule. There seems to be a marked variation in the action of the different strains of *Bact. abortivo-equinus* on this carbohydrate. This

variation is particularly marked in the heated media. In the unheated broth the reactions during the thirty day period are more uniform. The different strains do not show the same variation in the heated and unheated dulcitol media.

Inulin broth, which was fermented with a small amount of gas, shows also a very low initial acidity. Following this the inulin medium gradually becomes alkaline. This is perhaps to be explained in two ways. Inulin is often times impure and according to Hawk often contains a reducing sugar. Also inulin is hydrolyzed readily in the presence of an acid to levulose which carbohydrate is fermented by *Bact. abortivo-equinus* with the production of gas. The bubble of gas formed in melezitose may be due to an impurity in the sugar as the reactions in this media are similar to those in the other non fermented carbohydrates, i.e., a gradually increasing alkalinity.

It would seem that as a result of this work the following conclusions are justified.

1. *Bact. abortivo-equinus* does not form gas in lactose or sucrose. The apparent fermentations of these carbohydrates are often the result of hydrolysis in the sterilizing process.

2. *Bact. abortivo-equinus* does not ferment raffinose. Rhamnose is fermented with gas.

3. A gradually progressive acidity up to thirty days is produced by nearly all strains of *Bact. abortivo-equinus* in carbohydrate media where considerable gas is formed.

4. In carbohydrate broth in which no gas is formed a progressive alkalinity is formed over a thirty-day period.

5. In dulcitol media *Bact. abortivo-equinus* shows considerable variation in acidity and akalinity. This variation is found in unheated as well as heated media.

NOTE: Because of the necessity of economizing space it was deemed advisable to condense the fifteen tables originally prepared into one which is appended. This was done by computing the average titration figures for the eight strains. The variation in the acidity or alkalinity of the different strains was not large with the exception of their action on dulcitol. If readers desire we can furnish copies of the results obtained for each organism used.

Table showing the average action of eight strains of Bact. abortivo-equinus on the carbohydrates

SUGAR	INITIAL TITRATION	DAYS INCUBATED						GAS
		2	5	10	15	20	30	
								per cent
Heated glucose	0.6	2.9	3.2	3.6	4.1	4.5	6.0	23
Unheated glucose	0.4	3.4	3.7	3.9	4.1	4.5	5.8	20
Heated lactose	1.6	0.5	0.6	−0.5	−0.7	−0.9	−1.5	3
Unheated lactose	1.5	1.5	0.9	−0.6	−0.8	−1.0	−1.1	None
Heated sucrose	0.4	1.0	0.6	0.3	−0.4	−0.6	−1.1	None
Unheated sucrose	0.4	1.1	0.7	0.6	0.0	−0.8	−1.0	None
Heated maltose	2.2	2.2	3.5	4.2	4.5	4.5	5.0	35
Unheated maltose	0.7	2.7	3.2	3.7	4.4	4.5	4.8	29
Heated mannitol	1.1	3.1	3.3	3.4	3.5	3.8	4.5	70
Unheated mannitol	1.0	2.9	3.1	3.0	3.2	3.6	4.1	40
Heated mannose	1.4	2.9	3.2	3.4	3.7	4.1	4.2	50
Unheated mannose	1.4	2.9	3.1	3.3	3.5	3.7	3.9	50
Heated levulose	1.2	3.5	3.9	4.3	4.7	5.3	5.7	30
Unheated levulose	1.2	3.3	3.6	4.1	4.5	4.7	5.7	45
Heated dulcitol	1.5	3.0	3.2	3.3	1.0	0.6	0.8	63
Unheated dulcitol	1.5	2.2	2.5	2.3	1.9	3.0	3.0	40
Heated raffinose	1.1	1.6	−0.5	−0.7	−0.8	−1.1	−1.3	None
Unheated raffinose	1.8	1.4	0.7	−0.8	−1.1	−1.6	−1.7	None
Heated arabinose	1.8	3.1	3.2	3.7	4.4	5.3	5.8	30
Unheated arabinose	1.1	3.3	3.4	3.8	4.1	5.3	5.3	30
Heated rhamnose	1.7	3.4	4.0	4.5	4.8	5.6	6.0	40
Unheated rhamnose	1.5	3.7	4.1	4.6	3.5	4.4	4.0	35
Heated xylose	1.5	2.3	3.1	3.6	4.2	5.1	5.2	30
Unheated xylose	1.1	2.2	2.8	3.1	3.5	4.1	4.2	32
Heated melizitose	1.2	1.2	0.6	0.2	−0.2	−0.5	−0.5	0*
Unheated melizitose	1.0	1.0	0.2	−0.2	−0.6	−0.8	−0.8	0*
Heated inulin	1.7	1.7	0.9	−0.9	−2.2	−1.3	−0.8	3
Unheated inulin	0.6	1.0	0.5	−0.9	−2.2	−1.2	−1.3	5
Heated salicin	1.9	2.0	1.4	0.6	−0.2	−0.8	−0.9	None
Unheated salicin	1.6	1.5	1.0	0.9	−0.8	−0.9	−1.0	None

* Indicates size of the bubble of gas.

REFERENCES

DASSONVILLE AND REVIERE 1913 Contribution a l'etude de l'avortement epizootique. Rev. Gen. d. Med. Vet., **21**, 237

DE JONG, D. A. 1913 Uber einen Bacillus der Paratyphus B Enteritis gruppe als Ursache eines seuchenhaften Abortus der Stute. Cent. f. Bakt. Parastkunde u. Infek. I abt. Orig., **67**, 148.

GOOD, E. S., AND CORBETT, L. S. 1916 A study of gas-production by different strains of Bacillus Abortivo-equinus. Jour. Infect. Dis., **18**, 586.

MEYER, K. F., AND BOERNER, F. 1914 Studies on the etiology of epizootic abortion in mares. Jour. Med. Res., **29**, 325.

MUDGE, C. S. 1917 The effect of sterilization upon sugars in culture media. Jour. Bact., **2**, 403.

VAN HEELSBERGEN, T. 1913 Abortus bei Stuten durch einen Paratyphus B-Bacillus. Inaug. Dis. Utrecht.

BACTERIOLOGIC PEPTONE IN RELATION TO THE PRODUCTION OF DIPHTHERIA TOXIN AND ANTITOXIN

LEWIS DAVIS

From the Research Laboratory, Parke, Davis and Company, Detroit, Michigan

Received for publication April 5, 1920

I. INTRODUCTION

The necessity of using peptone media in the production of potent diphtheria toxin was recognized by the pioneer investigators in this field of serum therapy. Park and Williams (1896) appear to have been the first to lay any special emphasis on this constituent of the culture medium. After experimenting with varying concentrations of peptone in diphtheria toxin broth, they conclude that the strength of toxin averages greater with higher percentages of peptone (2 and 4 per cent) than with lower percentages (1 per cent). This is corroborated by Theobald Smith (1899), in his publication on the relation of glucose to the production of toxin in bouillon cultures of the diphtheria bacillus. He also proposed the use of a peptone bouillon in which the beef infusion, after preliminary reduction in acidity, was submitted to a fermentation with *Bact. coli*, in order to remove muscle sugar. Previous investigations by Spronck and von Fourenhout (1895) had indicated an inhibitory action of this carbohydrate upon the elaboration of toxin in peptone bouillon cultures of *Corynebact. diphtheriae*.

Martin (1898) attributed unsatisfactory results in toxin production to variations in composition of the peptone then available which appears to have been the Witte product. To overcome this, he proposed what he terms "liquid peptone" obtained from auto-digested swine stomachs which he mixed with an equal volume of veal bouillon. As would be expected, from its com-

position, this medium did not give successful results in the hands of other investigators, and found little application in practice. Spronck (1898) in the same year, recommended the substitution of a boiled and filtered extract of commercial yeast in place of the infusion of veal or beef. However, he specifically required the use of Witte peptone with his yeast product.

Subsequent publications on media for diphtheria toxin production appear to be more concerned with the reaction of the broth, and also, with the kind and condition of the meat used in the infusion. Veal is recommended by some in place of beef, while others insist that the meat before using must be either decomposed or fermented instead of freshly killed. This is in conformity with Theobald Smith's proposal for eliminating muscle sugar, which is assumed to interfere with toxin elaboration. Aside from the mention that the Witte product was employed, no attention appears to have been paid to peptone by later investigators.

The scarcity of Witte peptone during the past few years has again directed attention to bacteriologic peptone and to the methods of producing diphtheria toxin. A number of peptone products have appeared, of differing composition which, while allowing growth of the more common microorganisms and of *Corynebact. diphtheriae*, do not permit of obtaining the strong toxins formerly obtained. The use of trypsinized media as suggested by Cole and Onslow (1916) and the various modifications of Martin's peptone solution which have been recommended have not fulfilled practical requirements.

In the attempt to explain the necessary conditions for the successful application of some of the substitute products, rather unique views have been advanced concerning diphtheria toxin formation. Bunker (1919), in a recent article suggests "that there is a point at which toxin development is at its maximum, before which and after which the potency will be lost." He also states that "if time alone is made the basis of judging when toxin is 'ripe,' it will be only by chance and in spite of technique that any peptone will give satisfactory results." These statements as will be later shown, are contrary to extended practical experience in the routine production of high potency diphtheria toxin.

During the past year, the writer has had an opportunity to study large scale manufacture of diphtheria toxin. This included the preparation of thousands of liters of toxin having an L^+ dose of 0.33 cc., 0.25 cc., or less, and its use in horses for immunization purposes. It is the purpose of this article to consider the essentials for the routine production of high potency diphtheria toxin with special reference to its application in the development of high strength antidiphtheric serum.

II. THE PRODUCTION OF DIPHTHERIA TOXIN

a. Culture to be employed

Practically all of the institutions engaged in the propagation of *Corynebact. diphtheriae* for toxin elaboration employ the strain known as "Park-Williams bacillus no. 8" or "Culture Americana." Recently, six cultures of this strain were obtained from as many different laboratories. All were similarly carried by transplanting from two twenty-four-hour generations on Loeffler slant tubes and then in bouillon, according to technique later described. A decided variation in the strength of the final toxin, from the six cultures, was noted; two gave toxin of which one L^+ dose was greater than 0.5 cc. Only one, from the Hygienic Laboratory of the United States Public Health Service, gave toxin having an L^+ dose of 0.25 cc. or less. The toxins from two of the remaining three cultures gave L^+ doses of 0.50 cc. and the other one had a strength of $L^+ = 0.33$ cc. The advisability of verifying the toxicogenicity, particularly of any new strain of the organism is apparent. The parent culture is best maintained on slant tubes of moist Loeffler blood serum, transplants to bouillon being made as desired.

b. Culture medium

Of the various media recommended for toxin elaboration with *Corynebact. diphtheriae*, we have found plain beef infusion broth containing 2 per cent peptone and 0.5 per cent sodium chloride to be the most satisfactory. Extended trial with veal infusion in

place of the beef has not demonstrated any increase in toxicogenicity or shown any other advantages to warrant its use. Beef from various sources, including both cold storage and fresh products, has been employed in the infusion with equally satisfactory results. The increased acidity at times encountered with cold storage meat has not been found to require any special treatment. Preliminary fermentation of the infusion with a culture of *Bact. coli*, as first recommended by Smith (1899) to remove any fermentable carbohydrates is, at best, an unsatisfactory procedure, and in our experience, entirely superfluous. The formation, by the colon bacillus, of decomposition products which may give trouble on injection of the final diphtheria toxin, must also be taken into consideration.

Allowing the beef to infuse over night is not very practical with large scale preparation. Equally satisfactory results are obtained by adding twice the quantity of water to the minced beef and bringing to a boil in the steam kettle in the course of about an hour and a half. The resultant infusion liquor is then obtained by the use of a suitable press.

Fat must be eliminated from beef infusion, as even traces of fat have a decided inhibitory action on the production of diphtheria toxin. This has been demonstrated on numerous occasions when, out of the same lot of broth, those flasks which showed particles of fat gave final toxin of which the L^+ dose was greater than 0.5 cc., while the strength in the remaining flasks was 0.25 cc. or less. We are probably dealing here with a surface tension phenomenon, similar to that discussed by Larson, Cantwell and Hartzell (1919) in their recent paper on the influence of the surface tension of media on the growth of bacteria. It seems quite likely that the fat depresses the surface tension of the bouillon thus forcing *Corynebact. diphtheriae* to grow beneath the surface with resultant diminution in the formation of pellicle and toxin.

Experimentation with differing concentrations of peptone from 0.5 per cent to 4 per cent has shown that the most potent toxin requires a peptone content around 2 per cent. Amounts up to 4 per cent may be satisfactorily employed, but with no advantage over the smaller concentrations. The peptone used in

preparing the toxin under observation was Bacteriologic Peptone, Parke, Davis and Company, the composition and properties of which have been described in a previous article (Davis, 1917). Twenty grams of peptone and 5 grams of sodium chloride were added to every liter of beef infusion prepared as above, dissolved in the cold and then brought to a boil in the steam kettle to insure thorough solution.

Considerable uncertainty appears to exist as to what is the most satisfactory initial reaction for diphtheria toxin bouillon. Nearly all of the previous investigators, including Roux and Yersin (1888), Spronck (1898), Madsen (1897), Park and Williams (1896), Smith (1899) and Lubenau (1908) have employed either neutralization with litmus, which is crude at best, or "hot titration" with phenolphthalein, a procedure which is admittedly fallacious. The writer (Davis, 1918) in a paper on "Hydrogen ion concentration and toxicogenicity determinations with *Bact. diphtheriae*," has shown that potent toxin is produced in bouillon by *Corynebact. diphtheriae* only when the initial reaction falls within a certain zone of alkalinity, included within the hydrogen ion concentration limits of about 7.0×10^{-8} ($p\dot{H} = 7.2$) to about 5.0×10^{-9} ($p\dot{H} = 8.3$). The maximum degree of potency, however, is obtained when the reaction of the broth comes within the narrow limits of $p\dot{H} = 8.0$ to $p\dot{H} = 8.2$. This may be readily and consistently obtained by following the procedure given below for adjusting the reaction.

1. Transfer 10 cc. of the heated broth to a small Erlenmeyer flask and dilute with about 40 cc. of cold, distilled water. Add 0.5 cc. of a 1 per cent solution (95 per cent alcohol) of phenolphthalein as indicator and titrate to a deep pink color against an $\frac{N}{10}$ NaOH solution. The latter is preferably prepared when required as an exact 1/100 dilution of a $\frac{1\,0}{N}$ stock solution. The amount of the strong ($\frac{1\,0}{N}$) solution required to neutralize per litre of medium is given directly by the burette reading. Bring to a boil again in the steam kettle, and estimate the hydrogen ion concentration.

2. While the more accurate hydrogen electrode method is desirable for comparative and standardization purposes, equally

satisfactory results for routine production may be obtained by the colorimetric method. The simple "comparator" of Hurwitz, Meyer, and Ostenberg (1916) is recommended with standardized boric acid-potassium chloride-sodium hydroxide mixtures of pH = 8.0 and pH = 8.2, prepared as directed by Clark and Lubs. Exactly 10 cc. of the neutralized bouillon are transferred to a "comparison" tube, diluted with 10 cc. of distilled water, and mixed well. Ten cubic centimeters of the mixture are now removed to another tube and 0.5 cc. of an 0.02 per cent solution of phenolsulphonphthalein in 50 per cent alcohol next added. Two other tubes are prepared containing 10 cc. respectively of the standardized pH = 8.0 and pH = 8.2 mixtures, with 0.5 cc. of the phenolsulphonphthalein solution in each tube. The technique for comparison is as given by Clark and Lubs (1917). Usually, the value will be found to very closely approximate pH = 8.2. If, as may sometimes happen, the color in the tube containing medium plus indicator is lighter than the compensated pH = 8.0 standard, $\frac{N}{10}$ NaOH can be added directly to the former tube until the desired tint is reached. The burette reading, multiplied by two (since the equivalent of 5 cc. of the medium is used) gives the amount of $\frac{10}{N}$ NaOH required to correct each liter of broth.

Experience has repeatedly confirmed the observation that toxin of greater potency is obtained from broth contained in large flasks than in small ones. Other conditions being equal, we can expect to find diphtheria toxin of higher strength after growth in a six liter flask containing three liters of broth than in a liter flask containing 500 cc. Three liters of broth, dispensed into 6-liter, Florence type flasks have been employed in producing the toxin under observation.

It has undoubtedly been noted in every laboratory engaged in diphtheria toxin production, that, if for some reason, it becomes necessary to resterilize media, there is a resultant diminution in the strength of the final toxin. From experimentation in progress, to be reported upon in a later publication, it appears that food accessory factors, possibly of a vitamine character, are concerned in the production of diphtheria toxin. This makes it

especially desirable that the sterilization period of the medium should be as short as possible, to reduce destruction of the accessory factors to a minimum and yet be sufficient to ensure thorough heat penetration. Autoclave sterilization at 120°C. (15 pounds steam pressure) for a period not exceeding thirty minutes has given satisfaction.

c. Cultivation

The parent culture of *Corynebact. diphtheriae* on the Loeffler slants is transplanted through several twenty-four hour generations in tubes containing 10 cc. of bouillon to stimulate maximum pellicle formation The tubes are then used to inoculate small "starter" flasks containing 30 cc. of medium which are also incubated for twenty-four hours. The large flasks of broth are now inoculated with the twenty-four hour "starters," allowing one for each large flask. A temperature range of 36° to 38°C. has been found most satisfactory for incubation. Numerous potency tests have demonstrated that at least ten days incubation of the large flasks is necessary to ensure maximum elaboration of toxin and a twelve-day period is desirable. As has been shown in a previous publication (Davis, 1918), toxin of appreciable strength is elaborated by toxicogenic cultures within forty-eight hours. The potency gradually increases to a maximum value about the twelfth day, occasionally sooner. Incubation for an additional period of two weeks, or four weeks altogether, shows no deterioration of the final toxin. That this behavior is not confined specifically to the peptone employed is proved by the fact that, with the procedure as given above, Witte's peptone permits of similar results. In this case, toxin of maximum potency is obtained in the large flasks, only after a two-weeks' incubation.

When cultivated in plain bouillon under the optimal conditions already described, *Corynebact. diphtheriae* causes an initial increase in the hydrogen ion concentration of the medium. This is soon followed by a steady decrease until, apparently, a limiting alkaline reaction is attained. The following table, taken from the

article on hydrogen ion concentration determinations mentioned above (Davis, 1918) shows these changes with a toxicogenic strain.

Changes in H-ion concentration and toxicogenicity during growth of Corynebacterium diphtheriae in bouillon

TIME	C_H	pH	TOXICITY L^+ DOSE
hours			*cc.*
0	7.0×10^{-9}	8.15	
24	2.1×10^{-8}	7.68	1.0
48	3.2×10^{-8}	7.49	0.8
72	3.8×10^{-8}	7.41	
96	3.4×10^{-8}	7.47	0.55
120	2.1×10^{-8}	7.68	
144	2.5×10^{-8}	7.61	0.45
192	2.3×10^{-8}	7.64	
240	1.6×10^{-8}	7.79	0.15
312	8.7×10^{-9}	8.05	0.15
408	7.0×10^{-9}	8.15	0.15
528	5.0×10^{-9}	8.30	0.15

It is further shown in the same publication that the final hydrogen ion concentration of high strength toxin L^+ dose less than 0.25 cc.) after two weeks incubation ranged from $C_H = 1.6 \times 10^{-8}$ (pH = 7.79) to $C_H = 5.2 \times 10^{-9}$ ($pH^+ = 8.28$). At the same time, low strength toxins were obtained, the final H ion concentration of which came within the above limits. It is obvious from the table that in the normal development of *Corynebact. diphtheriae* in bouillon, the organisms may produce the same H ion concentration at two different intervals which represent wide variations in potency. This fact, and what has been stated above, justify the conclusion that there is no direct relationship, during or after growth, between the H ion concentration of the medium and production of toxin.

d. Final operations

The contents of the large toxin flasks, after proper incubation, are checked microscopically to determine purity of culture; 0.4 per cent of purified cresols is then added and allowed to act for, at least, twenty-four hours to ensure thorough germicidal action. As a rule, filtration can be accomplished satisfactorily through paper, otherwise Mandler filters may be employed. During

the above operations and in the finished condition, the toxin should be stored in a cool place.

It has been our experience in evaluating the strength of the diphtheria toxin for injection purposes, that the L^+ dose method (according to the Hygienic Laboratory) furnishes a more reliable index than determination of the minimum fatal dose. Although the theoretical relationship may not exactly hold, the L^+ dose for all practical purposes may be considered as 100 M. F. D.

III. GENERAL OBSERVATIONS

It will be readily conceded that we can best judge the value of what has been presented from the actual results of practical application. Data obtained during the past year in the production of several thousand gallons of diphtheria toxin by the general method indicated, are summarized below.

PRODUCTION RESULTS

Diphtheria toxin

Percentage of total toxin having L^+ dose

ABOVE 0.50 cc. (M. F. D. > 0.005 cc.)	0.50 cc. (M. F. D., 0.005 cc.)	0.33 cc. (M. F. D., 0.0033 cc.)	0.25 cc. OR LESS (M. F. D., 0.0025 cc. OR LESS)
10.2	11.3	35.5	43.0

As may be noted, practically 90 per cent of the toxin produced was of usable strength, $-L^+$ dose = 0.50 cc. or less. Of this, more than 78 per cent was high strength, having an L^+ dose of 0.33 cc. or less, and 43 per cent was so strong that one L^+ dose was 0.25 cc. or less. In view of the fact that only about 10 per cent of the large amount of toxin produced failed to reach a desirable strength, the procedure and medium recommended above can be considered as meeting practical requirements.

It is interesting to note, in connection with the production of diphtheria toxin, that no definite seasonal or weekly variation, as mentioned by MacConkey (1912) was observed. Occasionally, one or two bottles of a large lot would show an inferior or weak toxin, in spite of the fact that, as far as could be determined, the contents of all bottles after filtration should have been identical.

Data have been obtained in this study supporting the view that the troublesome local reactions encountered with horses in diphtheria treatment may be largely attributed to the method of controlling the hydrogen ion concentration of the toxin bouillon. It is a fact that in the use of the toxin under observation, practically no local reactions have been experienced. It is also true that adjusting the toxin bouillon by the colorimetric H ion method discussed above requires considerably less alkali than the use of the inaccurate "hot titration" method formerly employed. Whether it is the decreased amount of alkali used or possibly a diminished content of toxone bodies in the final toxin which is responsible for the favorable results must be left for further study.

Consideration of the foregoing production results would not be complete without data showing the antitoxic response to injection of the diphtheria toxin in horses. In the final analysis, this determines the utility of the toxin and consequently, the value of the methods and culture medium which are recommended. A summary has been prepared in the succeeding table to show the potency of the antidiphtheric serum obtained in the first yield from horses immunized during the past year with the toxin under discussion. The first large scale bleeding has been chosen for the valuation because experience has shown that the potency of this serum represents more closely than that from any of the succeeding bleedings the true value of the toxin injected.

Diphtheria antitoxin

Percentage of new horses yielding serum

UP TO 200 A. U.	200 A. U. TO 500 A. U.	500 A. U. TO 1000 A. U.	1000 A. U. TO ABOVE 1500 A. U.
22.4	27.7	34.3	15.6

Analysis of the table shows that nearly 78 per cent of all the new horses injected during the past year with the toxin under consideration produced a serviceable antidiphtheric serum (i.e., having a potency on first bleeding of 200 antitoxin units or greater). Eighty per cent of the productive horses (or 82 per

cent of the total number) gave serum ranging from 200 to 1000 units, 20 per cent (15.6 per cent of the total number) yielded the high potency products from 1000 to above 1500 antitoxin units, and 44 per cent (34.3 of all of the treated horses) came within the moderately high range from 500 to 1000 a.u. The foregoing and the fact that it has been possible in routine operation to immunize horses to an antitoxin strength exceeding 1500 units per cubic centimeter, can be taken as proof that the toxin used is fully satisfactory to meet all requirements of diphtheria antitoxin-production.

SUMMARY

1. A résumé of the more important literature on the production of diphtheria toxin is given This shows wide divergence of procedure. The recent scarcity of Witte peptone and failure of many substitute products to allow of appreciable toxin formation has further complicated the methods employed.

2. The essentials for the routine production of high potency diphtheria toxin are discussed. It is shown that, other conditions being the same, the toxicogenicity of the culture employed may vary within wide limits, according to the source. The necessity of verifying toxin production with any new strain is made apparent.

3. Plain beef infusion bouillon, containing peptone and salt is recommended for toxin production. Preliminary fermentation of the infusion with a culture of *Bact. coli* is shown to be undesirable. The use of veal infusion in place of beef is unnecessary. Even traces of fat must be avoided in the infusion as it interferes with maximum pellicle formation and thus diminishes toxin elaboration.

4. A content of 2 per cent peptone with 0.5 per cent of salt in the bouillon has been found to be most satisfactory.

5. Maximum strength of the final toxin has been obtained when the reaction of the broth comes within the limits of $pH^+ = 8.0$ to $pH^+ = 8.2$. A procedure is given for adjusting the hydrogen ion concentration to these values.

6. Cultivation for toxin production is best made in large flasks, previously inoculated with twenty-four hour cultures in

small "starter" flasks. Incubation is for at least ten days at 36°–38°C., with a twelve-day period preferred. Data are presented showing that the H ion concentration of the medium during growth cannot be used as an index of diphtheria toxin production.

7. Results are tabulated which have been obtained during the past year in the production of several thousand gallons of diphtheria toxin according to the procedure discussed. Practically 90 per cent of this toxin was of serviceable strength, $-L^+$ dose = 0.50 cc. or less. Of this more than 78 per cent was high strength, having an L^+ dose of 0.33 cc. (M. F. D. = 0.0033 cc.) or less, and 43 per cent had an L^+ dose of 0.25 cc. (M. F. D. = 0.0025 cc.) or less.

The efficiency of this toxin in the routine immunization of horses for antitoxin is shown by records of antidiphtheric serum production during the past year. Nearly 78 per cent of all new horses on this treatment produced serviceable antidiphtheric serum, i.e., having a potency on the first large scale bleeding of 200 antitoxin units or greater. Of the productive horses, 80 per cent gave serum ranging from 200–1000 units, 44 per cent yielded a product from 500–1000 units, and 20 per cent had serum coming within the very high range from 1000 to above 1600 antitoxic units per cubic centimeter.

REFERENCES

Bunker 1919 J. Bact., **4**, 379.
Clark and Lubs 1917 J. Bact., **2**, 1, 109, 191.
Cole and Onslow 1916 Lancet, **2**, 9.
Davis 1917 J. Lab. and Clin. Med., **3**, 75.
Davis 1918 J. Lab. and Clin. Med., **3**, 358.
Hurwitz, Meyer and Ostenberg 1916 Bull. Johns Hopkins Hospital, **27**, 17.
Larson, Cantwell and Hartzell 1919 J. Infect. Dis., **25**, 41.
Lubenau 1908 Arch. f. Hyg., **66**, 305.
Madsen 1897 Ztschr. f. Hyg. u. Infskr., **26**, 157.
Martin 1898 Ann. de l'Instit. Pasteur, **12**, 26.
MacConkey 1912 J. Hyg., **12**, 507.
Park and Williams 1896 J. Exp. Med., **1**, 164.
Roux and Yersin 1888, 1889, 1890, 1894 Ann. de l'Instit. Pasteur.
Smith 1899 J. Exp. Med., **4**, 373.
Spronck and von Fourenhout 1895 Ann. de l'Instit. Pasteur, **9**, 758.
Spronck 1898 Ann. de l'Instit. Pasteur, **12**, 700.

THE NOMENCLATURE OF THE ACTINOMYCETACEAE (ADDENDA)

R. S. BREED AND H. J. CONN

New York Agricultural Experiment Station, Geneva, New York

Received for publication April 13, 1920

Almost simultaneously with the publication of our previous paper with this title (Breed and Conn, 1919) our attention was called to the publication of an article by Merrill and Wade (1919). discussing the identical subject from a somewhat different viewpoint. The facts as given in the two papers supplement ach other, and both papers point out the fact that students of the group face a puzzling problem in regard to the proper name for the group. This arises because the general use of Streptothrix Corda, 1839 renders the term Streptothrix Cohn, 1875 invalid. This would make Actinomyces Harz, 1877 the valid name for the genus in question were it not for the fact that some hold that the use of Actinomyce by Meyen in 1828 invalidates Actinomyces Harz. In this case it appears that Discomyces Rivolta, 1878 becomes the correct name for the genus, although some argue that this name is also invalid.

Merrill and Wade have frankly accepted the principle of priority as final, and contend that Discomyces Rivolta is the only correct term to use, while we have quoted the International Botanical Code (Chap. III, Sect. 2, Art. 20 and Sect. 6, Art. 50) to show that under these rules it is necessary to regard general usage rather than priority in this case, and have suggested the adoption of Actinomyces Harz as a *genus conservandum*. After our paper was written, the Society of American Bacteriologists accepted this suggestion and have included Actinomyces Harz in their list of approved genera.

Since the publication of our paper, our attention has been called to the fact that the difference in spelling and derivation

between Actinomyce and Actinomyces is sufficient to cause them to be regarded as distinct under the International Botanical Code, Chapter III, Article 57.[1] We find that the same thing also holds true under the International Zoological Code, Article 36,[2] a fact that shows general and international acceptance of this view.

Although the stem words from which Actinomyce and Actinomyces are derived have identical meaning in the original Greek, "myce" is derived from the less commonly used feminine word, μῦκη, while "myces" comes from the masculine noun, μῦκης. Thus the two generic terms in question ought not to be regarded as homonyms as is done by Merrill and Wade. This view we find has already been expressed by Güssow (1914) in a paper which we had overlooked, and is confirmed by those authorities with whom we have consulted. This being the case legislative action by an International Congress is unnecessary. Actinomyces Harz is valid without such action and should be retained rather than Discomyces Rivolta.

REFERENCES

Breed, R. S., and Conn, H. J. 1919 The nomenclature of the Actinomycetaceae. J. Bact., **4**, 585–602.

Güssow, H. T. 1914 The systematic position of the organism of the common potato scab. Science, N. S., **39**, 431–433.

Merrill, E. D., and Wade, H. W. 1919 The validity of the name Discomyces for the genus of fungi variously called Actinomyces, Streptothrix and Nocardia. Philippine Jour. Sci., **14**, 56–69.

[1] "The original spelling of the name must be retained, except in the case of a typographic or orthographic error. When the difference between two names, especially two generic names, lies in the termination, these names are to be regarded as distinct even though differing by one letter only. Example: Rubia and Rubus, Monochaete and Monochaetum, Peponia and Peponium, Iria and Iris."

[2] "Recommendations. It is well to avoid the introduction of new generic names which differ from generic names already in use only in termination or in slight variation in spelling which might lead to confusion. But when once introduced, such names are not to be rejected on this account. Examples: Picus, Pica; Polyodus, Polyodon, Polyodonta, Polyodontas, Polyodontus."

PRELIMINARY NOTE ON THE USE OF SOME MIXED BUFFER MATERIALS FOR REGULATING THE HYDROGEN ION CONCENTRATIONS OF CULTURE MEDIA AND OF STANDARD BUFFER SOLUTIONS

M. R. MEACHAM, J. H. HOPFIELD AND S. F. ACREE

New York State College of Forestry, Syracuse University, Syracuse, New York

Received for publication April 13, 1920

In 1914–1915 Pieper (1917) began, with Humphrey and one of us, the use of a mixture of phosphoric and asparaginic acids in culture media and a number of other buffer mixtures were also employed. Pieper did not have our hydrogen electrode equipment set up to regulate the hydrogen ion concentrations, but this work was already planned. Meacham (1918) then began and has now finished investigations on this very important problem involving the relation between the rate of growth of fungi and the hydrogen ion concentration of culture media containing materials which enable any one now to regulate the hydrogen ion concentration at any desired value with great precision and reproducibility without using the hydrogen electrode or indicator methods. The result of his investigations on the growth of *Endothia parasitica*, *Lenzites sepiaria* and other organisms will appear in other articles.

It is desired to point out here the advantages to be derived by using a *single solution* of possibly two or three acids for giving a *smooth curve* relation between (a) the hydrogen ion concentrations from 10^{-1} to 10^{-14} and (b) the amount of alkali added to such mixture[1] of acids. The desirability of using[2] such a buffer mix-

[1] See also Prideaux (1916), whose excellent work was done after Pieper's but who did not suggest the use of his mixture with culture media. We did not discuss these physio-chemical relations in Pieper's article because he had no opportunity to regulate his media with the hydrogen electrode, this new important phase being taken up by Meacham in 1916–18.

[2] The mathematical side of the theory of buffer mixtures has now been developed in articles already completed, and applied in the measurement of the

ture is clear when we recall that the most important range of acidity in biological work is from a pH of 3 or 4 up to 8 or 9. But it is in just this region that we find the largest change in pH, *and therefore the largest errors,* when acid or alkali is added to the usual unbuffered culture media made from corn meal, beef, etc., which have small buffer properties. This is illustrated clearly in figure 1, where it is shown that, in adjusting a medium, an error of only 0.2 cc. of N/1 alkali in 100 cc. of corn meal extract

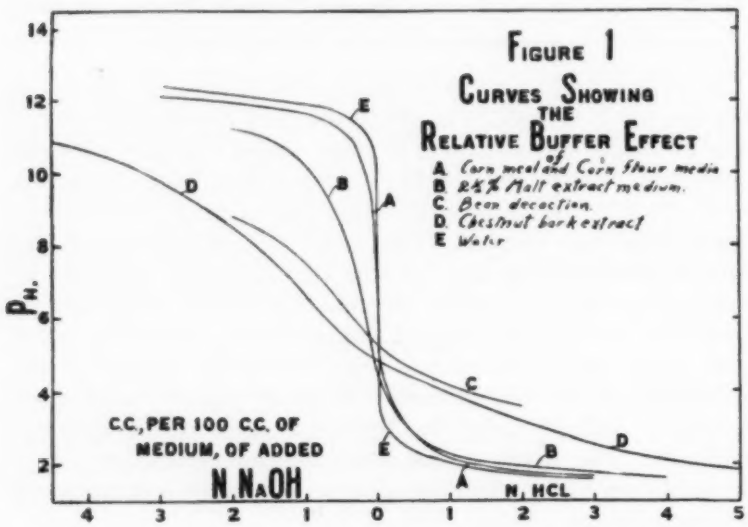


FIG. 1

changes the pH from about 4 to 10 and hence reduces the hydrogen ion concentration from 10^{-4} (or N/10,000) *to one-millionth that value,* or to 10^{-10} (or N/10,000,000,000). If the medium contains a mixture of buffer acids giving a smooth titration curve in this important biological range any desired acidity of the medium

ionization constants of asparaginic, phthalic, tartaric, glycerophosphoric, citric and pyrophosphoric acids. The theory is equally useful for analyzing a total titration curve back into the separate titration curves for each acid (or base) originally present or formed by the bacteria, or for calculating the total titration curve from the separate titration curves for the individual acids (or bases).

can be produced and maintained with small experimental errors, as illustrated in curves C and D in figure 1. In another article we are describing a large number of such useful acid and basic mixtures. For example, the mixture of asparaginic acid ($K_1 = 1.5 \times 10^{-4}$, $K_2 = 10^{-10}$) and of orthophosphoric[3] acid ($K_1 = 1.1 \times 10^{-2}$, $K_2 = 2 \times 10^{-7}$, $K_3 = 3 \times 10^{-12}$), used by Pieper in his synthetic medium gives nearly a straight line for the hydrogen ion concentrations when the equimolecular mixture of acids is treated with successive portions of alkali up to 5 molecules (fig. 2). This practically straight line is formed because a region of sharp inflection in the phosphoric acid titration curve, for example, is straightened out by adding another acid (or base) which, when it is 65 to 85 (especially when it is 75) per cent neutralized, gives a hydrogen ion concentration approximating that of the inflection point (midpoint) of the phosphate inflection curve. The various inflection curves of phosphoric (pyrophosphoric) acid and asparaginic acid are thus mutually nearly annulled. The slight deviation from a straight line will depend also upon the buffer properties of the other materials, if any, present in the medium, such as, for example, Witte's peptone, extracts

[3] Anhydrous sodium glycerophosphate is now sold as a buffer material which we believe will largely replace phosphoric salts for the following reasons. The glycerophosphate is less expensive than the highly purified potassium phosphate. It can be added in sterilized form directly to sterile culture media without producing the precipitate formed by phosphates. It does not precipitate out the calcium, magnesium and other heavy metals which are necessary for bacteria and can therefore be kept in solution with glycerophosphates and studied accurately in different concentrations. The titration curve is very close to that of phosphoric acid and enables workers to duplicate experiments previously buffered with phosphoric acid; the titration curve is furnished by the Grahame Chemical Company, 100 Rockingham Street, Rochester, N. Y., who sell glycerophosphates standardized for pathological and biological work. The glycerophosphates give the organisms a source of carbohydrate food in the glycerol residue which is apparently more readily assimilated than straight glycerol. Avery, Mellon and Acree have grown over 20 organisms, including tubercle bacilli, on solutions buffered with glycerophosphates. The glycerophosphates are stable enough to sterilize in accurately standardized tablets or in solution. The salt can be wieghed out and handled accurately as it is not hygroscopic like anhydrous sodium phosphate, for example. Sucrose-phosphate, mannitol-phosphate and other carbohydrate phosphates have the same desirable qualities and will be reported upon later.

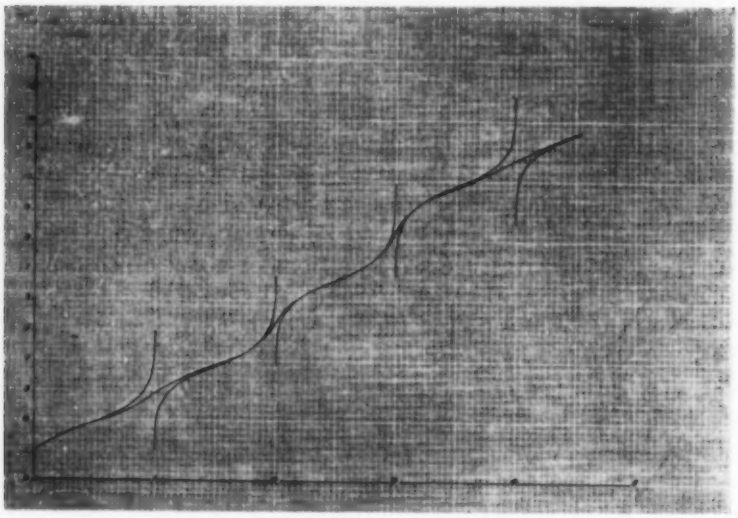


FIG. 2. SEPARATE TITRATION CURVES FOR ASPARAGINIC AND PHOSPHORIC ACIDS AND RESULTANT TITRATION CURVE FOR AN EQUIMOLECULAR MIXTURE OF THE TWO ACIDS

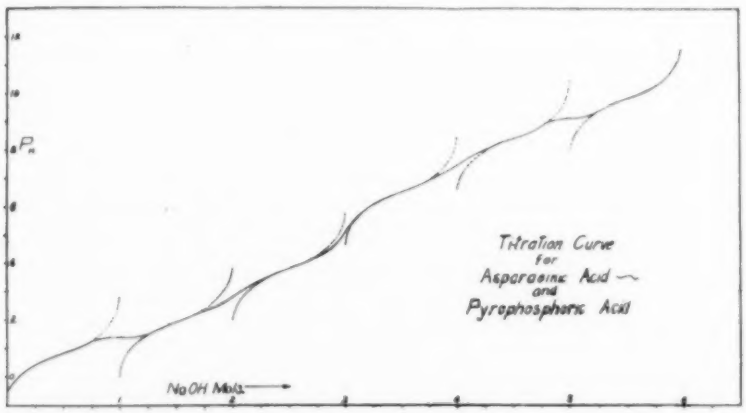


FIG. 3. SEPARATE TITRATION CURVES FOR ASPARAGINIC AND PYROPHOSPHORIC ACIDS AND RESULTANT TITRATION CURVE FOR AN EQUIMOLECULAR MIXTURE OF THE TWO ACIDS

of cornmeal, beans, malt, beef and other nutrient substances, as well as upon the acid or basic products formed by the fungi. An equimolecular mixture of asparaginic (or aminomalonic or acetoacetic) and pyrophosphoric acid ($K_1 = 1.4 \times 10^{-1}$, $K_2 = 1.1 \times 10^{-2}$, $K_3 = 3 \times 10^{-7}$, $K_4 = 3.6 \times 10^{-9}$) also gives nearly a straight line relation between the hydrogen ion concentration and the number of molecules of alkali added (fig. 3). The use of pyrophosphoric acid in this connection is to be recommended for the reason that Bray and Abbott (1909) have found the ionization constants of the third and fourth acid groups to have the above mentioned *unusual* values. It is clear that there would be nearly a straight line relation between the number of molecules of alkali added and the hydrogen ion concentration between about 10^{-6} and 10^{-10}. This is a very important region in the study of seawaters and many natural waters and other solutions which are slightly alkaline and it is doubtless familiar to many that there are practically no organic carboxylic acids having ionization constants between 10^{-6} and 10^{-9} or 10^{-10}. The second acid group of maleic acid has an ionization constant about 3×10^{-7}, and the second acid group of benzylmalonic acid has an ionization constant 4.9×10^{-7}. Outside of phenols and similar substances, there are no organic acids with ionization constants below these values until we come to the aminocarboxylic acids such as asparagin, aminoacetic acid, etc. with ionization constants around 10^{-9} and 10^{-10}. An inorganic substance like pyrophosphoric acid is therefore very welcome to cover this very important range and it has the added advantage that it is not readily decomposed by bacteria or fungi as are the organic acids. Furthermore it does not precipitate out the calcium and magnesium from natural waters or culture media. The only apparently undesirable feature of pyrophosphoric acid is that the work of Abbott (1909) has shown that in acid solutions pyrophosphoric acid is hydrolyzed into the orthophosphoric acid and that the rate of hydration increases with increasing hydrogen ion concentration. It is therefore certain that in very acid culture media containing pyrophosphoric acid there would be a change into orthophosphoric acid. It is clear, however, that there could be

no very great change under average conditions for bacteriological work around 25°C. because the hydrolysis is fairly slow and the first acid group of orthophosphoric acid has an ionization constant about equal to that of the second acid group of pyrophosphoric acid (1.1×10^{-2}). As work with solutions more acid than $C_H = 10^{-2}$ will be rare, the hydrolytic cleavage (of anions and molecules) can be represented by the equation:

$$NaH_3P_2O_7 + H_2O \rightarrow NaH_2PO_4 + H_3PO_4$$

From this equation and the above ionization constants it is seen that there is no appreciable change in the *hydrogen* ion concentration. The preparation of mono-, di-, tri-, and tetra-salts from pure pyrophosphoric acid and the rate of hydrolytic cleavage of each will be studied carefully in this connection. The change of the tetra-salt into the orthophosphate by the action of alkalies will also be studied.

It is highly desirable that the hydrogen electrode be employed more widely in the study of the ionization constants of such acids and in the study of the acidity of such regulated culture media *before and after* the growth of organisms on such media. Professor Abbott and Professor Bray have most generously consented to our work in this field as accessory to their own investigations, which are practically the only authoritative records of the ionization constants of these acids, especially pyrophosphoric acid. We consider their work to be of a very high order of merit and it is hoped that further studies in the application of these materials in culture media and the studies by use of the hydrogen electrode and spectrophotometric methods will be of importance.

It should be pointed out that the use of the above mixture of asparaginic (aminomalonic, acetoacetic, etc.) acid and orthophosphoric acid (or pyrophosphoric acid) or equivalent mixtures simplifies very greatly the work involved in measuring the useful ranges of indicators, comparisons with culture media, and related fields. This applies to both the buffer solutions[4] alone and those

[4] The Grahame Chemical Company now sells a sterile single buffer solution which remains constant indefinitely and covers all pH values from 1 to 14 very accurately and replaces the 5 or 6 buffer solutions employed heretofore. They also

used in culture media. The buffer solutions are sterilized with thymol, formaldehyde, chloramides, etc. Heretofore investigators interested in these lines have had to use *several different solutions* to get hydrogen ion concentrations between 10^{-2} and 10^{-12} for example. As the preparation of each solution requires considerable care and some of these materials are decomposed by molds, fungi and bacteria, the solutions had to be renewed every few weeks. From our work and that of Prideaux we believe that *one solution* containing asparaginic[5] acid and orthophosphoric (or pyrophosphoric) acid in equimolecular quantities suffices to cover the entire range between 10^{-1} and 10^{-12} in practically a straight line or smooth curve relation between the hydrogen ion concentrations and the number of molecules of alkali added to the mixture of the two acids. We also avoid the use of the boric acid employed by Prideaux which is undesirable in most biological work. The buffer or *culture solutions can be made and kept sterile by making the original solutions decidedly alkaline at ordinary temperatures instead of in autoclaves and afterwards adding a (sterile) strong acid in known amounts to secure solutions having higher hydrogen ion concentrations.* This method is advantageous when the alkali or acid and other materials present interact and change the hydrogen ion concentration when heated in an autoclave.

In making a titration curve on such a strongly akaline solution of the salts of two acids (in equimolecular proportion) we recommend the feature of adding to the alkaline solution in the hydrogen electrode vessel another solution containing the asparaginic and phosphoric acids (in equimolecular proportions and in

furnish sterile buffer tablets, with or without admixed standardized quantities of different indicators, which cover all pH values from 1 to 14 in steps of 0.2 pH. These sterile tablets are added to sterile culture media or to water to give the desired pH values, and have been found very useful in saving time and securing accurate results.

[5] The asparaginic acid can be replaced very advantageously in culture media by $\frac{1}{2}$ equivalent of formic acid, $\frac{1}{2}$ equivalent of acetic acid, and one equivalent of aminoacetic or equivalent acid. For buffer solutions this aminoacetic acid can be replaced by phenolsulphonic acid, thymol, or other suitable and stable substances having ionization constants around 10^{-10}.

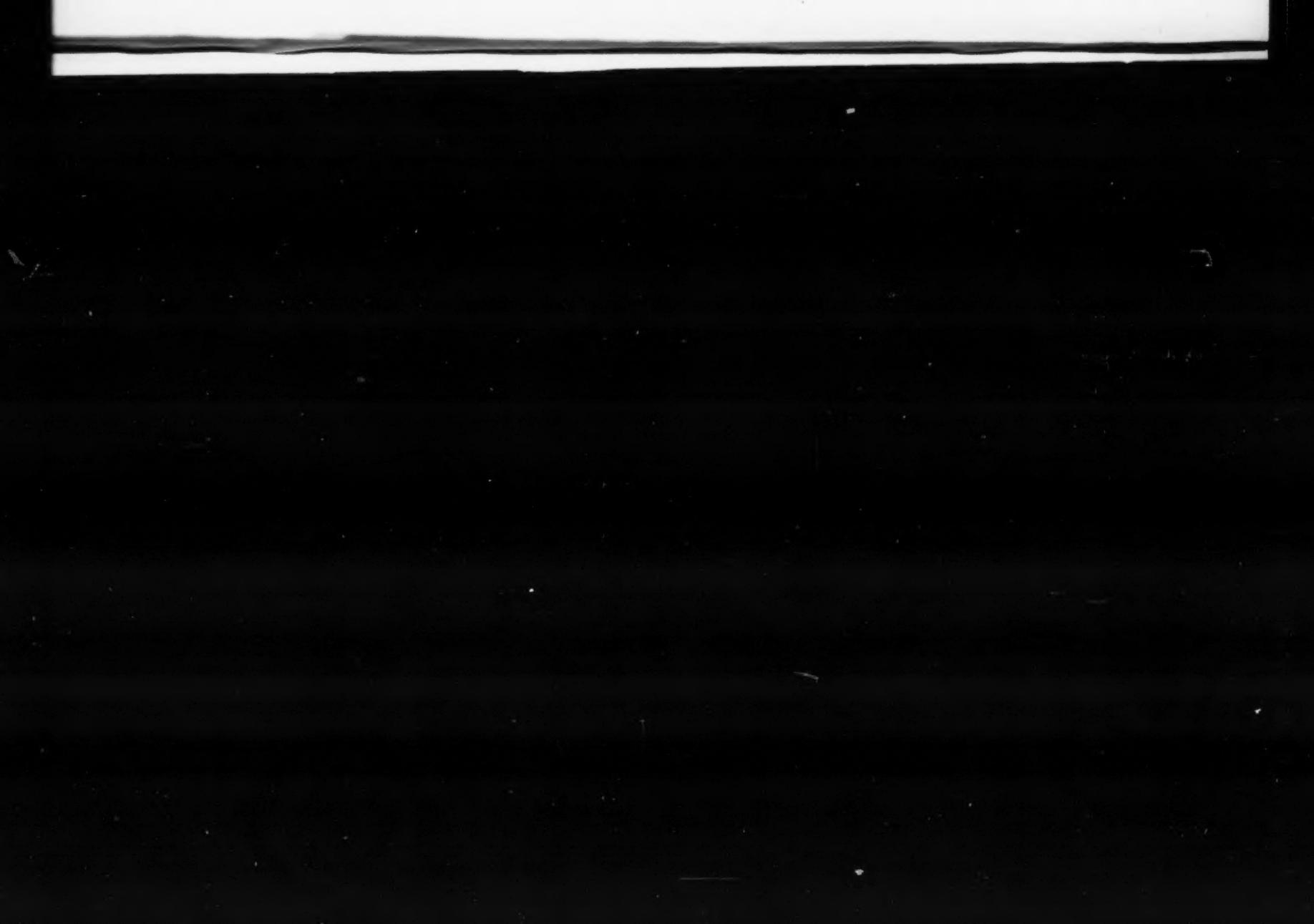
the same concentration employed in the first solution) and containing also any desired amount of hydrochloric or other strong acid necessary to liberate any part or all of the asparaginic and phosphoric acids. It is clear that in this way we can keep the concentration of the asparaginic and phosphoric acid (or the salts of these) constant at any desired concentration, say M/10, and at the same time change the hydrogen ion concentration from that of the very alkaline solution up to that of the very acid solution obtained when a sufficient volume of the solution of hydrochloric, asparaginic and phosphoric acid is added to the alkaline solution. It is furthermore clear that a similar procedure would enable the operator to make a choice of the constant concentration at which he could keep the *cations* and *anions* in making such a titration curve. Without further discussion, it is clear that the *one solution* of the mixture of phosphoric and asparaginic acids could be made to give any desired hydrogen ion concentration by simply adding varying known quantities of alkali and water to keep the solutions standard. Likewise, the one solution of disodium (or potassium) asparaginate and tripotassium (or sodium) orthophosphate could be made to give any desired hydrogen ion concentration by simply adding calculated quantities of hydrochloric acid. The above described method of mixing a very alkaline and a very acid solution of equimolecular parts of asparaginic and phosphoric acid (in say M/10 concentrations of each acid in both solutions) will perhaps be a more convenient way of making a titration curve and of getting any desired hydrogen ion concentration by mixing the proper volumes of the two solutions. The hydrogen ion concentration is measured easily by means of the hydrogen electrode, a standard calomel or hydrogen *comparison* electrode and the Loomis-Acree (1911) method of eliminating contact potentials by the use of 4.1N potassium chloride. The errors of the Bjerrum extrapolation method are discussed in another article.

Mr. J. H. Hopfield has completed a titration curve on asparaginic acid from the very acid to the very alkaline regions. The ionization constants of the two acid groups and the basic group have been calculated from his data and will be reported in a

separate article. Miss Margaret Brennan has studied the growth of fungi on such regulated media in continuation of Dr. Meacham's work. It is hoped that other investigators will leave this field to us for a reasonable time so that we shall have an opportunity to complete certain phases of the work which will then be published in detail.

REFERENCES

BRAY AND ABBOTT 1909 Jour. Amer. Chem. Soc., **31**, 729. ABBOTT: Ibid., **31**, 763.
LOOMIS AND ACREE 1911 Jour. Am. Chem. Soc., **46**, 585, 621.
MEACHAM 1918 Science, **48**, 499. MEACHAM, HOPFIELD AND ACREE: This Jour., **5**, 305, 309 (1920).
PIEPER 1917 Phytopathology, **7**, 214.
PRIDEAUX 1916 Proc. Roy. Soc. A, **92**, 463.



A MUTATING, MUCOID PARATYPHOIDBACILLUS ISOLATED FROM THE URINE OF A CARRIER

TH. THJØTTA AND ODD KINCK EIDE

From The Bacteriological Laboratory of the Norwegian Medical Corps, Kristiania

Received for publication March 30, 1920

Mutating strains of paratyphoidbacilli are mentioned several times, especially by Baerthlein who has described different forms of colonies and variants in respect to the size of the bacilli. Recently a mutating form was described by Fletcher who found that the stool of a carrier who was examined at regular intervals suddenly became almost free of the typical colonies of paratyphoidbacilli. Simultaneously he found a new form of bacillus that he had not been aware of before. This microbe formed large wet colonies on the agar and did not agglutinate in a paratyphoidserum. Fletcher considered the microbe a mutating form of paratyphoid and called it mucoid on account of its wet mucuslike consistency.

With the exception of Fletcher the earlier authors on the subject of transmutation in paratyphoidbacilli do not seem to have observed many alterations as to the agglutination in mutating bacilli, and in the case of Fletcher this author does not bring forth any explanation of the lack of agglutinability. We have therefore thought it worth while to present an account of a paratyphoid strain, that we have watched while mutating and made the object of a fairly minute examination.

This strain was isolated from the urine of a man whose record of sickness is as follows:

In December, 1918 he contracted paratyphoid fever of the ordinary type. In January of the following year he had a severe attack of pyelitis of paratyphoid origin. This first attack was followed by numerous others and the patient developed a condition of chronic pyelitis with a continuous discharge of paratyphoidbacilli in his urine. The

strain of bacilli was quite typical until the month of May, when we observed that a new type of colonies became dominant in the cultures. The main characters in which these differed from the ordinary colonies were their large size, their shiny, wet aspect and their lack of agglutinability. The new type was present in great numbers while the ordinary type was markedly reduced. The condition became quite stabile and was the same all the summer and autumn, when the patient passed out of our observation.

The strain studied by us was isolated from the urine in September, 1919, and has now (March, 1920) the same characters as it had immediately after isolation from the patient.

On the surface of litmus agar it forms large, wet, shiny colonies that are much elevated over the surface and have a regularly rounded circumference and top. If growing very close together they flow into each other, forming large irregularly shaped figures, that may be taken to be isolated colonies of a peculiar aspect, but in reality are formed from several colonies melted together. These appearances have led Fletcher to talk about colonies of ameboid shape, an expression that is certainly justified, if it is remembered that these figures are not isolated colonies. Viewed by transmitted light the colonies have a very dense mass that has a distinct reddish hue. Left outside the incubator for some days the colonies, sometimes, dry up, and sink together and the color deepens into a clear blue. In this stage they look more like true paratyphoid than in a fresh condition, when their appearance is distinctly against this diagnosis.

Cultivated in carbohydrates the atypical strain ferments the sugars just as a paratyphoidbacillus should do, and it does not form indol in broth. Examined under the microscope in living condition, the bacilli of the atypical strain are as a rule quite immobile. Here and there is seen a bacillus showing slight movement, in no respect like the common vivid motions of the paratyphoidbacilli. In fixed and dyed films the bacilli are exceedingly small, often being chained together like diplobacilli or even like diplococci.

Testing the agglutinability of these bacilli, we found that they completely lacked the ability to be agglutinated in the prelimi-

nary test directly from the culture test carried out in a drop of serum on a slide. We therefore immunized a rabbit with the atypical strain and another with the normal strain from the same patient. The agglutination was titrated in the serum of the patient.

The result of these tests are as follows:

1. Agglutination test in the patient's serum (kindly recorded by our friend Dr. Kr. Skajaa):

Atypical strain 1:320
Typical strain 1:320

After absorption with a typical Para. B strain:

Atypical strain ..... 1:20+
Para. B strain ..... 1:20+

After absorption with the atypical strain:

Atypical strain ..... 1:20+
Para. B strain ..... 1:80+

The serum of the patient thus agglutinates the atypical as well as the typical strain. After absorption the agglutination is abolished or diminished both in case of the atypical and a typical paratyphoid strain.

2. Agglutination in immunesera from rabbits.

TESTED STRAINS	SERUM PRODUCED WITH THE STRAIN								
	Atypical			Normal			Para. B		
	Titer on reading after hours								
	2	24	48	2	24	28	2	24	28
Atypical	400	800	3200	800	1600	6400	400	800	3200
Typical	800	1600	1600	1600	1600	6400	800	1600	3200
Para. B	1600	3200	3200	1600	1600	3200	1600	6400	6400

After absorption during 24 hours with the three strains atypical, typical and Para. B 16, the sera showed the following titres:

TESTED STRAINS	ABSORPTION WITH ATYPICAL IN HOMOLOGOUS SERUM	ABSORPTION WITH NORMAL IN HOMOLOGOUS SERUM	ABSORPTION WITH PARA. B IN HOMOLOGOUS SERUM
Atypical	100	200	200
Typical	200	200	800
Para. B 16	800	400	1600

These tests were carried out in cultures from well isolated colonies, but not with one cell cultures. As it, however, was thought advisable to exclude the possibility of our working with cultures containing both atypical and typical bacilli, we prepared one cell cultures after the method of Burri, and made the following serological tests with cultures, that had been obtained from but one atypical bacillus, and that in growth were just as atypical as the cultures had been before.

a. Agglutination in paratyphoidserum:

	2 hours	6 hours
Typical strain	1600	6400
Atypical strain	800	1600

b. Absorption test after Castelliano with atypical strain in 6 hours (antigen added twice):

Agglutination titer after six hours in the incubator

Typical strain	400
Atypical strain	200

c. Complement absorption test:

Antigen. One fiftieth of a twenty-four hours old culture (agar slope), emulsified in saline and killed by heat.

Serum. Paratyphoidserum produced with a standard paratyphoid B strain. Dose: 0.05–0.0001 cc.

Complement. Fresh guinea-pig serum, dose: 0.06 cc.

Time of absorption. Six hours at 8°.

Blood corpuscles of sheep, washed and emulsified as in the test of Wassermann.

Hemolytic amboceptor. As in test of Wassermann.

SERUM	RESULT	
	Atypical strain	Typical strain
cc.		
0.05	Inhibition	Inhibition
0.025	Inhibition	Inhibition
0.0125	Inhibition	Inhibition
0.0063	Inhibition	Inhibition
0.0032	Inhibition	Inhibition
0.0016	Marked inhibition	Marked inhibition
0.0008	Trace of inhibition	Hemolysis
0.0004	Hemolysis	Hemolysis
0.0002	Hemolysis	Hemolysis
0.0001	Hemolysis	Hemolysis
0.0		
Control of antigen	Hemolysis	Hemolysis

The tests show with certainty that the atypical strain is a real paratyphoid strain. Injected into a rabbit it has caused the production of a serum that agglutinates two quite typical paratyphoid strains: and after absorption of two sera with typical strains the titre of agglutination is lowered practically to the same degree in all these three strains. The tests show however that the new strain is much slower in its agglutinating reaction than either of the two typical strains. In all tests the former required forty-eight hours to reach its maximum of agglutination, while this is reached in about twenty-four or even in two hours by the typical ones. Consequently the atypical strain is not less agglutinable than the typical ones, but is slower in responding. This will, however, in practice amount to the same thing, because a strain that does not show an agglutination test in a given time on isolation, especially if this happens in a strain with quite atypical colonies, will be set aside as not being paratyphoid, and thus a carrier will not be discovered.

After having shown that the atypical strain is a real paratyphoid strain, we will have to answer the question, why it shows this peculiar growth. It will already be observed that there are several characters about this strain that point to a special group of microbes, namely the capsule-producing bacteria. The *Bact. pneumoniae* of Friedlaender, for instance, produces such large,

wet colonies, and the bacilli of this group are small bacilli without any motility and also lack agglutinability. These facts rendered it necessary to examine our strain with a capsule dye. It was a fairly easy task to demonstrate great masses of mucus in slides, prepared from our cultures. The bacilli were not surrounded by small distinct capsules as is the case in pneumococci. The capsules were very thick, surrounding one, two or many bacilli in a common cover of mucus. Between the heaps of bacilli there were great masses of mucus, arranged as a homogeneous intercellular substance. This marked formation of mucus might also be demonstrated directly in the cultures, as these were quite sticky and often in agarslope cultures produced mucus that flowed down into the bottom of the tube.

At this point in our investigation we happened to come across a strain of *Bact. pneumoniae* in a case of pneumonia in a guinea-pig. We could now compare the two strains, and we have the greatest difficulty in telling which of the strains was the paratyphoid and which the pneumonia bacillus seen on the plates.

We consequently consider it proved that our atypical strain has obtained the faculty of forming capsules or rather of producing great masses of mucus. This explains very well the peculiar growth, the small bacilli, the slight motility and the slow agglutinability. The strain is of a special interest as it has been watched during its growth in typical manner in the urogenital tract of the patient and has been seen to develop into the capsule producing variety.

It looks as if our strain must be considered a true transmutation of the paratyphoid bacillus. The strain developed the new character quite suddenly and has kept it unaltered for more than ¾ year. It thus appears to be very constant, but it would be of interest to show that it kept unaltered even if the strain was cultivated rapidly for several generations from the one directly to the next without being allowed to stand over in the laboratory between the cultures. We consequently secured a well isolated colony and spread it on agar plates. After twenty-four hours of growth a new colony was taken out, spread again and this was done daily for three weeks. The first few cultures showed a

very few typical colonies that in turn were propagated to ascertain whether they kept their typical aspect or reverted to the atypical one. This, however, was not the case. From a typical colony only typical ones grew in the subcultures. On the other hand, when the atypical colonies had been transferred for a few times the fully developed colonies showed only the atypical aspect. At the end of three weeks we had to deal with a culture that never showed any sign of being a mixture of the two kinds of colonies, and since has always kept its character.

Thus it is shown that the atypical strain kept its new character unaltered when propagated rapidly from one artificial medium to the next. We thought it necessary to see if it would also keep this character when living under pathogenic conditions. For this experiment we used white mice, that were very susceptible to inoculations with these microbes. After intraperitoneal injections of small amounts of living bacilli the animals died in about twenty-four hours. From each mouse some blood was taken and injected into a new one without being cultivated on artificial media first. As a control of the bacilli at hand cultures were simultaneously made on litmus agar. After being injected into 8 mice the microbe still gave only atypical colonies in cultures.

As we thought it to be of some interest to find out if the virulence of the atypical strain had altered with the new character we inoculated several white mice with measured volumes of agar slope cultures of the atypical and the typical strain of the bacillus and watched the time when the mice died.

The result of this experiment was as follows:

	AMOUNT OF CULTURE							
	1 loop	1/10	1/20	1/50	1/100	1/200	1/500	1/1000
	Mouse died within hours							
Typical strain....	20	20	20	20	30	24	Lives	Lives
Atypical strain...	8	24	24	24	Lives	Lives	Lives	Lives

It will be noted that the typical strain kills the mice in smaller doses than the atypical strain. It must, however, be remem-

bered, that the same volume of the atypical strain cannot contain so many bacilli as the typical one, because the mucus produced by the former will take up a space that in the typical strain is filled by bacilli. It is thus obvious that the same amount of culture will contain more bacilli in the typical than in the atypical strain. We cannot therefore expect to find the same titer of virulence in the two strains, unless the virulence of the atypical strain has increased after the transmutation. This is not the case in our strain, the virulence being either the same or slightly diminished.

It has been pointed out that the new character was quite stable as well under saprophytic as under pathogenic circumstances. We are, therefore, justified in calling the alteration of our strain a true transmutation. Why this had developed is a very interesting question which however is, we believe, not solvable. The new character might be looked upon as an effort by the bacillus to obtain a higher resistance against the serumbodies of the probably highly immune patient. In this connection we will point out the fact that increased formation of capsules has been seen in bacteria simultaneously with a strengthened resistance and virulence. Thus Sauerbeck talks about an immunity of the bacilli through a structural adjustment, while Gruber and Futaki have found that weakened anthrax bacilli were not capable of producing such large capsules as the full virulent strains.

In order to try whether our atypical strain might show any difference in its resistance against serum as compared with the typical strain of our patient we carried out bactericidal tests in active human serum with both strains. The result was the following:

SERUM	ATYPICAL STRAIN, COLONIES	TYPICAL STRAIN, COLONIES
cc.		
0.25	0	10
0.125	20	50
0.063	100	Several 1000
0.032	Several 100	∞
0.016	∞	∞
0.008	∞	∞
0.004	∞	∞

The test consequently shows that the capsule producing strain is not the more resistant of the two strains, rather the contrary. This result is in full accordance with our virulence test. If the resistance had been increased in the capsule forming strain it would have been natural that the virulence should have increased also, as the bacilli in the small non-lethal doses in this case would have had the opportunity of growing to a number sufficient to kill before they had been eliminated by the serum of the animal.

The importance of the observation recorded in this paper is mainly in the value it may have for epidemiological practice. It is namely obvious that transmutations of the kind related may hinder the detection of a paratyphoid carrier, as the investigator will pass over colonies of an appearance so unlike paratyphoid-colonies, and especially, if he finds that they do not agglutinate in a specific serum.

Theoretically it is of some interest that we have watched the origin of the transmutated colonies in the urine of the patient as Fletcher already has seen a similar strain arise in the stool of another patient. The two cases considered together give evidence that such transmutations may occur in the alimentary as well as in the urogenital tract of patients.

CONCLUSIONS

The urine of a patient suffering from cystopyelitis quite suddenly showed numerous colonies of an atypical bacterium, while the typical paratyphoid colonies were much reduced in number. The new microbe turned out to be a capsule producing, mucoid paratyphoid strain with the following characters:

1. It grew in large, very wet, slimy colonies with a reddish grey hue.
2. The bacilli were small, the motility slight or lost.
3. The agglutinability was specific, but the reaction was very slow, the maximum reached in a far longer time than was the case with typical strains.
4. The absorption of complement took place exactly in the same doses and in the same time as in tests with the typical strain.

5. The microbe showed the same resistance against normal human serum as the typical strain. Likewise white mice were killed by nearly the same doses of the two strains.

This microbe satisfies all claims that are necessary to establish a real transmutation of bacteria.

REFERENCES

BAERTHLEIN: Ergebnisse der Immunitaetsforschung, Experimentellen Therapie, Bakteriologie und Hygiene, **1**, 68 (by Weichardt).

FLETCHER: Capsulate, mucoid forms of paratyfoid and dysentery bacilli. The Lancet, July 27, 1918.

GRUBER AND FUTAKI: Lehrbuch d. Pathogenen Mikroorganismen, **1**, 583.

SAUERBECK: Ibid., p. 61.

THJØTTA, TH.: Et tilfælde av paratyføs cystopyelit. Medicinsk Revue, 1919.

BACTERIOLOGIC STUDIES IN CHRONIC ARTHRITIS AND CHOREA[1]

J. H. RICHARDS

Departments of Clinical Pathology and Medicine, Cornell University Medical College, New York City

Receivedfor publication March 22, 1920

I. BACTERIOLOGICAL STUDIES IN ARTHRITIS

This work was part of a study of chronic arthritis undertaken to determine whether any constant cultural or immunologic streptococcus was associated with the disease. At the outset it was found necessary to define clearly the type of the disease studied on account of the ever changing terminology due to etiologic studies, and consequent changes in classification. For instance, one case of gonorrhoeal arthritis may simulate a case of acute rheumatic fever, and another an arthritis deformans. A complete bacteriologic study is necessary for a proper classification, and the bacteriologic studies in arthritis are by no means yet finished. Therefore any classification now given will have to be changed as the result of subsequent studies. However, so that the type of case studied may be somewhat defined, it may be well to state in a broad way a possible general classification.

ARTHRITIS

Non-bacterial in origin

a. Traumatic
b. Metabolic including gout, acute and chronic
c. Following injection of diphtheria antitoxin and other sera
d. Secondary to nervous diseases, e.g., tabes
e. Associated with certain diseases, e.g., scurvy, hemophilia, purpura, etc.

[1] Presented before the Society of American Bacteriologists, December 31, 1919.

Associated with bacteria in origin

a. Acute rheumatic fever

b. Acute suppurative arthritis, e.g., acute pyogenic joint infection.

c. Chronic suppurative arthritis, e.g, tuberculosis of joint.

d. Acute non-suppurative arthritis, e.g., that acute exacerbation which occurs frequently during the course of a chronic non-suppurative arthritis. These attacks are usually mildly febrile, and present a local picture not unlike that of acute rheumatic fever. The joint involved may be one previously diseased, or may be a newly involved one.

e. Chronic non-suppurative arthritis

1. Syphilitic. This condition is much more common than is recognized and is frequently found complicating other types

2. A type of arthritis probably not representing a disease entity, but rather a group of diseases which have been variously termed:

Arthritis deformans, Virchow
Rheumatoid arthritis, Garrod
Osteo arthritis, Garrod
Rheumatic gout, Fuller
Chronic rheumatic arthritis, Adams
Nodosity of the joints, Haygarth, etc.

The dominant features of this type are the tendency to involve many joints especially the smaller ones, the disposition to the destruction of the articular and periarticular tissues resulting in exostoses and ankylosis, and the usual progressive chronicity of the disease.

It is this latter type that was selected for this study. It remains for investigators to determine by etiologic studies whether this type represents a chronic form of acute rheumatic fever, or whether in this type there is included a group of diseases. Also if a constant bacterium is found associated with the disease, it must be determined whether the arthritis is the result of the activity of the bacteria themselves in the articular or periarticular tissues, or the result of toxines elaborated by these bacteria in some remote focus. This work makes no pretense of clearing up these questions, for the streptococcus studied is not

always found, and it has not been determined that the disease is produced by the inoculation of the streptococcus or its toxins. Further work on this subject is now under way.

The literature of this subject is not satisfactory on account of the variations of classification. Very little bacteriologic work has been done on this type. D. J. Davis (1913) found that in this class of case streptococci could rarely be demonstrated excepting at autopsy, but he did occasionally obtain this organism from the regional lymph glands. E. C. Rosenow (1914) found that by making cultures directly from the glands draining the inflamed joints in 54 cases there could be isolated

	Cases
Modified *Streptococcus viridans*	32
Staphylococcus	5
Bact. mucosum	3
Neisseria catarrhalis	1
Gonococcus	1

Moon and Edwards (1917) in 83 cases of chronic arthritis took blood cultures using the technique described by Rosenow and obtained

	Cases
Negative cultures	58
Non-hemolytic streptococcus	18
Bact. mucosum	3
Diphtheroid bacillus	3
Unidentified	1

In acute rheumatic fever much work with not at all uniform results has been done, notably by Poynton and Paine (1913), Beattie and Yates (1912-13), R. I. Cole (1914), H. K. Faber (1915), Swift and Kinsella (1917), E. C. Rosenow (1914), Moon and Edwards (1917) and others. It is difficult to determine what types of cases have been included in these studies. Very frequently cases of typical chronic deforming arthritis at the outset present the picture of acute rheumatic fever; and acute rheumatic fever may extend for months with a low grade fever relapsing into a severe type for a few hours or days at each new focal extension, and terminating either in death, complete recovery, or recovery with damaged tissues and a tendency to relapse. On account of the frequency of these border line cases, the blood

cultures for the present study were taken from patients who were constantly afebrile excepting for the infrequent attacks of fever, lasting for a short time only, during an exacerbation of the arthritis, and the cultures were not taken at the time of the fever. No case is included in this series that did not have at least three months' history of arthritis. It was purposed to include no case that could possibly be considered as acute rheumatic fever. Likewise no case is included with a history of syphilis or a positive Wassermann reaction, or a history of a recent gonorrhoea or a positive gonorrhoeal fixation test.

The technique of blood culture was the same in all cases:

1. North's medium was used. Heating this medium for sterilization caused different batches of the media to increase in acidity to markedly different degrees. It was found necessary for the development of the streptococcus obtained, to titrate the medium so that after the final heating at the time of planting, the reaction was 0.3 to 0.5 $\frac{N}{10}$ acid, using phenolphthalein as an indicator.

2. Five to ten poured plates were used, and planting was done immediately after taking the blood.

3. The concentration of the blood in the plates was varied, so that the first plate had in it only about 0.3 cc. of blood, and the last 5 to 6 cc. This procedure showed that very often a growth would be obtained in the dilute plates and none at all in the more concentrated ones. It may be that the whole blood itself acts to inhibit the development of the growth. It is well known that whole blood makes every attempt to rid itself of bacteria, and does not lend itself to use as a medium as well as other tissues of the body.

4. Incubation of the plates was continued for ten days. To prevent drying, the plates were inverted as soon as they had set, and were then placed over a basin of water in the incubator. The development of the original culture did not appear in two cases until the eighth day of incubation, and the average time was five days. For this reason, some procedure was necessary to prevent drying of the plates, for under the usual conditions the plates dried on the third or fourth day.

5. Transplantation was made as soon as the earliest colony appeared, for in the original culture, there seemed to be only a low degree of viability. More frequently than otherwise no transplant was obtained after waiting for the culture to develop fully. Up to a certain point these cultures seemed to increase in viability by transplantation, but the majority of our cultures did not survive six or eight transplantations.

6. The character of the growth in the original blood culture was not always typically that of *Streptococcus viridans*. Only three produced green in the original culture, but the remaining 11 did so in the transplants. These original colonies appeared like little black spots that could be seen only in a good light. In addition to the fourteen positive blood cultures herein reported we obtained six additional cultures which we were unable to transplant. These colonies were of the type just described; they retained the Gram stain, and presented on the slide the morphology of the other colonies which were transplanted and identified.

7. Identification of the transplants was done by Dr. Thro. All transplants had the same cultural characters:

Did not break up in bile
Did not peptonize milk
Grew in chains in broth
Produced green in blood
Retained the Gram stain
Were of low grade virulency to rabbits

Blood cultures were taken in 104 cases and this organism was found in 14 cases, and no bacteria but this organism were found in any culture. The organism in question can be classed as *Streptococcus viridans*. Control cultures were made from the blood of other patients and no growth of this organism was obtained.

Complement fixation tests with *Streptococcus viridans* antigens were done in all cases. The technique of complement fixation was based on the work of Schwartz and MacNeil (1911) in their study of complement fixation in gonorrhoeal infections, and on the

work of Swift and Thro (1911) in their study of streptococci by the use of complement fixation. The difficulties of the test lie in the preparation of efficient antigens. The one that has given most satisfactory results in our hands is the unkilled, thoroughly washed, salt ground bacterial emulsion. In our comparative experiments this method gave a greater difference between the fixing power and the anticomplementary dose than the Besredka dried antigen, the heated and killed bacterial emulsion, or the extracted filtered antigen. Swift and Thro found the fixing power of an extract made from dried bacteria highest compared to the anticomplementary power. At the beginning of this work, several antigens (5 to 9) prepared from different strains of *Streptococcus viridans* were used. Hastings (1914) found the necessity for the employment of many antigens to be due to the fact that a group reaction could not be obtained. We also found this necessary when working with an antigen of high anticomplementary action. But when an antigen was prepared as just described a polyvalent antigen of pooled strains all from Arthritis deformans gave satisfactory results on account of the higher fixing power—anticomplementary ratio. This is illustrated by the results of case 25 on the table appended. An autogenous antigen was not found more efficient than this polyvalent antigen excepting in case 6. In this case we obtained no inhibition excepting with an antigen prepared from an autogenous tooth culture. For the past two years our bacterial vaccines have been standardized by their nitrogen content. This has been found much more accurate than the Wright method of counting, Of late it has been found convenient to determine a working dilution of our antigens by this method, and usually the fixing power and the anti-complementary dose could be determined in a dilution containing 0.000028 gram bacterial nitrogen per cubic centimeter.

Joint cultures were attempted in 54 cases and *Streptococcus viridans* was obtained in 4 cases. These cultures were taken during an acute exacerbation, and an attempt was made to obtain fluid from the inflamed periarticular tissues as well as from the joint cavity.

TABLE 1

Determination of anticomplementary and fixing power of different antigens with serum from case 25

SERUM CASE 25	ANTIGEN	A	B	C	D	E	F	G	H	I	
cc.	*cc.*										
0	0.05	++++	++++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.06	0+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.07	0+++	++++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.08	0+++	0+++	++++	++++	++++	++++	+++	++++	++++	++++
0	0.09	0 0++	0+++	++++	++++	++++	++++	++++	++++	++++	++++
0	0.10	0 0++	0 0++	0+++	++++	++++	++++	++++	++++	++++	++++
0	0.11	0 0 0 0	0 0 0+	0+++	++++	++++	++++	++++	++++	++++	++++
0	0.12	0 0 0 0	0 0 0 0	0 0++	++++	++++	++++	++++	++++	++++	++++
0	0.13	0 0 0 0	0 0 0 0	0 0 0 0	++++	++++	++++	++++	++++	++++	0+++
0	0.14	0 0 0 0	0 0 0 0	0 0 0 0	++++	++++	++++	++++	++++	++++	0+++
0.02	0.01	0 0 0 0	0+++	0 0++	0+++	0 0 0+	0 0++	0+++	0 0 0 0	0 0 0+	0+++
0.02	0.02	0 0 0 0	0 0++	0+++	0 0 0+	0 0 0 0	0 0 0+	0 0++	0 0 0 0	0 0 0+	0+++
0.02	0.03	0 0 0 0	0 0++	0 0++	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0 0 0+	0+++
0.02	0.04	0 0 0 0	0 0++	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0 0 0+	0 0++
0.02	0.05	0 0 0 0	0 0++	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0++	0 0 0 0	0 0 0 0	0 0 0+
0.02	0.06	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0+
0.02	0.07	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0+
0.02	0.08	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0 0	0 0 0+	0 0 0 0	0 0 0 0	0 0 0 0

++++ = complete hemolysis.
0+++ = about 75 per cent hemolysis.
0 0++ = about 50 per cent hemolysis.
0 0 0+ = about 25 per cent hemolysis.
0 0 0 0 = complete inhibition.
A = Washed, unkilled, bacterial emulsion of pooled strains.
B = Washed, killed by 60°C. one hour, bacterial emulsion of pooled strains.
C = Besredka extract of pooled strains.
D = Washed, salt ground bacterial emulsion of pooled strains.
E = Washed, salt ground bacterial emulsion of strain 1.
F = Washed, salt ground bacterial emulsion of strain 2.
G = Washed, salt ground bacterial emulsion of strain 3.
H = Washed, salt ground bacterial emulsion of strain 4.
I = Washed, salt ground bacterial emulsion of strain 5.
J = Washed, salt ground bacterial emulsion of autogenous strain.

The feces of all cases was examined by Gram stain, and those that showed streptococci on the slide were cultured. Streptococci were found by the Gram stain in 42 cases, and were isolated and identified as *Streptococcus viridans* in 5 cases. This is an interesting phase of the work and we are continuing work along this line. The technique of making these cultures was as follows: Select a loopful of clear mucus, wash thoroughly in saline, and plant several plates very thinly. Even by this method we had great difficulty in obtaining the five positive cultures on account of the rapid overgrowth of other bacteria found in the stool.

The technique of culture of the various foci is as follows:

Teeth. When a sinus or pyorrhoea was present a culture was taken directly from the pus. This pus was usually found to contain two or more types of bacteria. When a tooth was withdrawn a culture was taken from the root and the socket, and frequently *Streptococcus viridans* was found in pure culture.

Tonsil. After gargling with sterile saline a loop was inserted as deeply as possible into a crypt of the tonsil using a pillar retractor when necessary for a clear view. If the tonsils were removed a culture was made from the deeper portions through an incision of the tonsillar capsule.

Ethmoid and sphenoid cultures were taken from the discharges from these sinuses after cleansing the nasal cavity with sterile salt solution. The material to be planted was taken as nearly as possible from the opening of these sinuses. In cases 3 and 7 the cultures were taken directly from the sinuses during an operation by E. Ross Faulkner. The other nasal sinuses were cultured in a similar manner.

Prostate. After urinating, the prostate was massaged and the culture made directly from the discharge.

Pyelitis. The sediment from a freshly catheterized urine was immediately planted on North's medium.

Salpingitis. The one case was cultured at the time of operation.

From a study of the table herein appended giving a summary of the 104 cases it is found that

1. In blood cultures *Streptococcus viridans* was found in 14 cases out of 104 cases.

TABLE 2

Review of cases of arthritis

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI
1	M.	38	+	+		0	+	Chronic endocarditis	+	+	
2	M.	41	+	+		+	+		0	+	
3	M.	40	+	+	+	0	+	Acute rheumatic fever	+	+	Ethmoids, sphenoids
4	F.	44	0	+	0	0	+	Chronic endocarditis	+	+	
5	F.	49	+	+		0	+		+	+	
6	F.	44	+	+	0	0	+		+	0	
7	M.	50	+	+		0	+		+	0	Ethmoids, maxillary
8	F.	52	+	+			0		0	+	
9	F.	48	+	+			0		+	0	
10	M.	49	+	+	0	+	+		+	+	
11	F.	46	0	+	+	+	+		0	+	
12	F.	58	+	+	0		0		+	+	
13	M.	39	+	+	0		0	Chronic endocarditis	0	0	Prostate
14	F.	36	+	+	0	0	+		0	+	
15	M.	40	0	0	0		0	Chronic endocarditis, acute rheumatic fever	0	0	
16	M.	39	0	0	−	+	+		+	0	
17	F.	46	0	0		0	+	Acute rheumatic fever	+	0	
18	F.	35	0	0	0	0	+		0	+	
19	M.	47	+	0			0		+	+	
20	M.	39	+	0	0		0	Acute rheumatic fever	0	0	
21	M.	51	0	0		0	+		+	0	
22	F.	52	+	0		0	+		0	0	
23	F.	49	+	0			0		0	+	
24	F.	42	+	0			0	Chronic endocarditis	0	0	
25	F.	44	+	0			0		+	+	

TABLE 2—*Continued*

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI.
26	M.	49	+	0		0	+		0	0	
27	M.	47	0	0	0	0	+		+	0	Ethmoids
28	M.	60	+	0	0	0	+		+	0	
29	M.	62	+	0	0	0	+		0	0	
30	M.	59	0	0	0	0	+		+	+	
31	F.	48	+	0		0	+		0	+	
32	F.	38	+	0		0	+		0	+	
33	M.	47	+	0	0		0		0	0	
34	M.	36	0	0	0	0	+		+	0	
35	F.	29	+	0	0		0		0	0	
36	M.	52	+	0	0		0		0	0	
37	F.	54	0	0	0	0	+		0	0	
38	M.	30	+	0	0	0	+		0	0	
39	M.	38	+	0	0		0		0	0	Ethmoids
40	M.	44	0	0	0		0	Acute rheumatic fever	0	0	
41	M.	36	+	0	0		0	Acute rheumatic fever	+		
42	M.	38	+	0	+		0		+	0	
43	M.	61	0	0			0	Chronic endocarditis	+	+	
44	F.	57	+	0			0		+	0	
45	F.	55	+	0	0		0		+	+	
46	F.	53	0	0	0	0	+		+	0	
47	F.	46	+	0		0	+		+	0	
48	F.	48	+	0		0	+		0	0	
49	F.	57	+	0			0		+	+	
50	M.	46	+	0			0	Acute rheumatic fever	0	0	
51	M.	51	0	0			0		0	0	
52	F.	42	+	0	0	0	+		+	+	
53	F.	51	+	0			0		+	0	
54	M.	52	+	0	0		0		+	+	
55	M.	41	0	0			0	Chronic endocarditis	+	+	
56	M.	43	+	0	0		0		0	0	

TABLE 2—*Continued*

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI
57	M.	45	+	0			0	Acute rheumatic fever	0	+	
58	M.	52	+	0	0		0	Chronic endocarditis	+	0	
59	M.	53	+	0	0	0	+		0	+	
60	M.	45	0	0	0		0		0	+	
61	M.	47	+	0		0	+		0	0	
62	M.	51	+	0		0	+		+	0	
63	F.	48	0	0	0	0	+	Acute rheumatic fever	+	0	
64	F.	39	+	0		0	+		0	0	
65	F.	64	0	0			0		+	0	
66	F.	48	0	0	0		0		0	+	
67	F.	62	0	0			0		0	0	
68	F.	11	+	0			0	Chorea	+	0	Ethmoids, sphenoids
69	M.	19	+	0			0		0	0	
70	F.	28	+	0			0		0	0	
71	M.	42	0	0	0		0		0	+	
72	F.	41	+	0		+	+		+	0	
73	M.	15	+	0			0		+	0	
74	F.	35					0		+	0	
75	F.	36	0	0	0	0	+	Chronic endocarditis	+	+	
76	M.	34	+	0	0		0	Acute rheumatic fever	0	0	
77	M.	48	0	0			0		+	0	
78	M.	51	+	0			0		0	0	
79	M.	39	+	0			0		0	0	
80	F.	33	+	0	0	0	+	Chronic endocarditis	+	+	
81	F.	46	+	0	+		0		+	+	
82	F.	44	+	0	0		0	Acute rheumatic fever	+	+	Maxillary
83	M.	41	0	0			0		+	0	
84	F.	17	0	0			0	Chorea	0	0	

TABLE 2—*Concluded*

CASE NUMBER	SEX	AGE	STREPTOCOCCUS VIRIDANS COMPLEMENT FIXATION	STREPTOCOCCUS VIRIDANS BLOOD-CULTURE	STREPTOCOCCUS VIRIDANS JOINT-CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	PAST HISTORY OF CHOREA-ACUTE RHEUMATIC FEVER CHRONIC ENDOCARDITIS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	OTHER STREPTOCOCCUS VIRIDANS FOCI
85	F.	28	0	0		0	+		0	0	
86	M.	39	0	0	0		0		0	0	
87	M	44	0	0	0		0		0	0	
88	F.	52	+	0		0	+		0	+	
89	F.	52	+	0		0	+		+	0	
90	F.	68	+	0			0		+	0	
91	F.	38	0	0	0		0		0	+	
92	F.	37	0	0	0		0		0	0	Kidney-pelvis
93	F.	39	+	0	0		0	Chronic endocarditis	+	+	
94	M.	44	0	0	0		0		+	+	
95	M.	46	+	0	0		0		+	0	
96	F.	37	+	0	0	0	+		0	0	
97	M.	41	+	0	0	0	+		0	+	
98	F.	36	0	0			0		0	+	
99	F.	42	+	0	0		0		0	0	
101	F.	50	+	0	0		0		0	+	
102	F.	36	+	0	0		0		0	+	
103	F.	48	0	0			0		0	0	
104	F.	39	0	0			0	Acute rheumatic fever	0	0	
100	M.	46	+	0	0		0		+	0	Prostate

In all cases Wassermann and gonococcus fixation tests were negative.

2. Chronic endocarditis was found in 11 cases.
3. A history of acute rheumatic fever was obtained in 12 cases.
4. A history of chorea in 2 cases.
5. Foci of *Streptococcus viridans* infection were found:

	Cases
Teeth	50
Tonsils	40
Ethmoid	5
Frontal	0
Sphenoid	2
Antrum	4

	Cases
Prostate	2
Pyelitis	1
Salpingitis	1

6. Complement fixation with *Streptococcus viridans* antigen was found positive in 68 cases.

7. Joint fluid cultures demonstrated *Streptococcus viridans* in 4 out of 54 cases.

8. No foci of *Streptococcus viridans* infection were found in 20 cases, and of these 20 negative cases 11 gave positive complement fixation tests. In addition to these 20 cases, there were 9 cases in which a streptococcus was seen in the Gram stain of the stool but could not be obtained in culture for identification, and of these, 7 gave positive complement fixation tests with *Streptococcus viridans* antigen.

TABLE 3

Review of cases of chorea

CASE NUMBER	SEX	AGE	WASSERMANN	GONOCOCCUS FIXATION	STREPTOCOCCUS VIRIDANS FIXATION	STREPTOCOCCUS VIRIDANS BLOOD CULTURE	STREPTOCOCCUS VIRIDANS FECES-CULTURE	STREPTOCOCCUS FECES GRAM-STAIN	ENDOCARDITIS	HISTORY ACUTE RHEUMATIC FEVER	PRESENT INFLAMMATION JOINTS	STREPTOCOCCUS VIRIDANS FOCUS TEETH	STREPTOCOCCUS VIRIDANS FOCUS TONSIL	STREPTOCOCCUS VIRIDANS FOCUS NOSE	GONOCOCCUS FOCUS VAGINA	DURATION OF CHOREA
1	F.	13	0	0	+	0	0	+	+	0	0	+	+	0		11 days
2	F.	9	+	0	+	0	0	+	+	0	0	0	+	0		5 weeks
3	F.	14	0	0	+	0	+	+	0	0	+	+	+	0		7 days
4	F.	15	0	0	+	0	0	+	0	+	0	+	+	+		2 weeks
5	F.	11	+	0	0	0	0	0	+	0	+	0	0	0		2 weeks
6	F.	11	0	+	0	0	0	0	0	0	0	0	0	0	+	9 days
7	F.	18	0	0	+	+	+	+	0	0	0	+	+	+		4 weeks
8	M.	9	0	0	+	0	0	0	+	0	0	0	0	0		8 weeks
9	M.	8	0	0	+	+	0	0	+	0	+	0	0	0		10 days
10	M.	14	0	0	+	+	0	+	+	0	0	+	0	0		7 weeks
11	F.	14	0	0	+	0	0	+	+	0	0	+	+	+		4 weeks
12	F.	10	0	0	+	+	+	+	+	0	+	+	0	0		11 days
13	F.	10	0	0	+	+	0	+	+	0	0	0	+	+		15 days
14	F.	13	0	0	+	0	0	0	0	0	0	0	0	+		5 days
15	M.	9	0	0	+	0	0	0	+	0	0	+	+	0		18 days
16	F.	19	0	0	0	0	0	0	0	+	0	0	+	0		11 weeks

II. BACTERIOLOGIC STUDIES IN CHOREA

In January 10, 1913, I reported two cases of chorea in which *Streptococcus viridans* was found in the blood. Since that time I have studied 16 cases in addition. The technique of cultures and complement fixation was the same as was used in the bacteriologic studies in chronic arthritis just reported. In cases 2 and 5 the Wassermann reaction was positive In case 2 there were no other signs of lues, and on account of the absence of both parents, hereditary lues could not be established. Case 5 had an interstitial keratitis, and the mother gave a positive history of lues contracted from the father. Case 6 gave a positive gonococcus fixation test, and was found to have a history of eight months leucorrhoea in which gonococci were found. In this case we could find no focus of *Streptococcus viridans* infection, and the complement fixation was negative with *Streptococcus viridans* antigen. Altogether in 4 cases no focus of *Streptococcus viridans* infection was found. The complement fixation with *Streptococcus viridans* antigen was positive in 13 cases, and negative in 3. The blood cultures showed *Streptococcus viridans* positive in 5, and negative in 11 cases. The feces cultures showed *Streptococcus viridans* in 3 cases, but Gram stain streptococci were found in 9 cases. Other foci of *Streptococcus viridans* infection were found in

	Cases
Tonsils	9
Teeth	8
Nose	5

All cultures were isolated and identified in the manner just described in the bacteriologic studies of chronic arthritis.

REFERENCES

BEATTIS AND YATES 1912–1913 J. Path. & Bact., **17**, 538–551.
BESREDKA 1904 Ann. de l'Inst. Pasteur, **18**, 363.
COLE, R. I. 1914 J. Infect. Dis., **1**, 714–737.
DAVIS, D. J. 1913 J. A. M. A., **61**, 724–727.
FABER, H. K. 1915 J. Exp. Med., **22**, 615–638.
HASTINGS, T. W. 1914 J. Exp. Med., **20**, no. 1.
MOON AND EDWARDS 1917 J. Infect. Dis. Chi., **21**, 154–161.
NORTH, C. E. 1909 J. Med. Research, **20**, 359.
POYNTON AND PAINE 1913 Complete works. London, p. 109.
ROSENOW, E. C. 1914 J. Infect. Dis., **14**, 61; J. A. M. A., **63**, 905.
SCHWARTZ AND MACNEIL 1911 A. J. Med. Sci., **141**, 693.
SWIFT AND KINSELLA 1917 Arch. Int. Med., **19**, 381.
SWIFT AND THRO 1911 Arch. Int. Med., **7**, 24–27.